Ovarian ageing:

Clinical and molecular derangements during reproductive ageing in women

- a new glimpse of an old problem

Mette Haug Stensen

Department of Gynecology

Oslo University Hospital

Faculty of Medicine

University of Oslo

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“You woke up this morning to what is effectively a 29-hour day. Twenty-four of those hours, you will use now; the other five will be put by for later”. Thomas B.L. Kirkwood

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List of papers

Paper I

Routine morphological scoring systems in assisted reproduction treatment fail to reflect age-related impairment of oocyte and embryo quality.

Stensen MH, Tanbo T, Storeng R, Åbyholm T, Fedorcsak P.
Reproductive Biomedicine Online. 2010 Jul; 21(1):118-25

Paper II

Advanced glycation end products and their receptor contribute to ovarian ageing.

Stensen MH, Tanbo T, Storeng R, Fedorcsak P.
Human Reproduction. 2014 Jan; 29 (1):125-34

Paper III

Fragmentation of human cleavage-stage embryos is related to the progression through meiotic and mitotic cell cycles.

Stensen MH, Tanbo TG, Storeng R, Åbyholm T, Fedorcsak P.
Fertility and Sterility. 2015 Feb; 103 (2):374-381

Abbreviations

AGE	advanced glycation end products
AGE-BSA	advanced glycation end products - bovine serum albumin
AMH	anti-Müllerian hormone
ART	assisted reproductive technology
Aur-B	aurora B kinase
BSA	bovine serum albumin
CML	carboxymethyl-lysine
COH	controlled ovarian hyperstimulation
CRP	C-reactive protein
ECM	extracellular matrix
EGA	embryonic genome activation
FACS	fluorescence-activated cell sorting
FFDC	follicle fluid-derived cells
FSH	follicle stimulating hormone
GLC	granulosa-lutein cell
GV	germinal vesicle
hCG	human chorionic gonadotrophin
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
MI	metaphase I
MII	metaphase II
MG	methylglyoxal
mtDNA	mitochondrial DNA
MTOC	microtubule organizing center

NF-κB	nuclear factor-κB
ORT	ovarian reserve test
PARP	poly-(ADP-ribose)-polymerase
PB1	polar body I
PBMC	peripheral blood-derived mononuclear cells
PCOS	polycystic ovary syndrome
PI	propidium iodide
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PN	pronuclei
POR	poor ovarian response
PS	phosphotidylserine
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen spices
RPL	recurrent pregnancy loss
SAC	spindle assembly checkpoint
Sgos	shugoshins
sRAGE	soluble receptor of advanced glycation end products
TAGE	toxic advanced glycation end products
TE	trophectoderm

Summary of papers

Paper I

Routine morphological scoring systems in assisted reproduction treatment are based on parameters that presumably correlate with the biological quality of gametes and embryos, including chromosome abnormalities. Maternal age is a key factor predicting pregnancy and live birth, and it is therefore of considerable interest to identify age-related indicators of oocyte and embryo quality. The purpose of Paper I was to examine whether routine morphological scoring systems reflect age-related impact on oocyte and embryo quality among 4587 couples undergoing their first assisted reproduction treatment. The study assessed over 43,000 oocytes, 25,000 embryos and 7900 transferred embryos and analyzed the associations among the following parameters: number of oocytes retrieved, oocyte quality, including maturity, fertilization rates, embryo quality, based on morphological features, and treatment outcome. Advanced chronological age was found to be associated with fewer oocytes retrieved, fewer embryos available for cryopreservation, as well as lower pregnancy, implantation, live birth rates and a higher miscarriage rate. No age-related correlation was found between fertilization rates, oocyte or embryo quality. Routinely used morphological scoring systems, such as assessment of blastomere count, shape and fragmentation, fail to reflect age-related impact on oocyte and embryo quality.

Paper II

Advanced glycation end-products (AGEs) are formed by non-enzymatic glycation of proteins, lipids, and DNA. AGEs are involved in the pathogenesis of diabetes mellitus, cardiovascular disease, and Alzheimer's disease. In reproductive ageing, intra-ovarian accumulation of AGE may induce oxidative stress-response through interaction with multi-ligand transmembrane receptors for AGE (RAGE). Accumulation of ageing-related molecular damage in ovarian somatic cells may also stimulate immune responses contributing to decline in ovarian function. In Paper II we aimed to determine whether follicular fluid-derived cells express AGE and the receptor RAGE and characterize downstream effects of RAGE activation, by immunofluorescence microscopy and multi-color flow cytometry. Both ovarian granulosa-

lutein cells (GLC) and ovarian monocytes were found to express AGE and RAGE on the cell surface. RAGE-specific fluorescence intensity in GL cells correlated with the patients' chronological age. GL cells, monocytes and lymphocytes were found to bind AGE-BSA, and the binding of AGE-BSA of GL cells correlated with the patients' chronological age. Binding of recombinant human chorionic gonadotrophin (rhCG) to GL cells was not affected by the presence of AGE-BSA. In GL cells, AGE-BSA and BSA failed to induce cleavage of caspase-3, phosphorylation of NF-κB, and increased binding of annexin V. AGE-fibronectin was found to induce detachment of cultured GL cells *in vitro*. The findings support that ovarian granulosa cells and monocytes are exposed to AGEs *in vivo*, express RAGE, and bind AGEs on the cell surface. The ligands of RAGE and their effects in the ovary remain uncertain. Structural long-lived extracellular matrix (ECM) proteins rather than soluble AGEs may play a role in the decline of ovarian function during ageing.

Paper III

Cytoplasmic fragments are often observed during the first few cells divisions of developing human embryos *in vitro.* The fragments may contain genetic material, even whole chromosomes, and a high degree of embryo fragmentation predicts inferior development. The mechanisms involved in embryo fragmentation remain unclear, but given the association between fragments and aneuploidy, it has been hypothesized that fragmentation is related to temporal derangements during the cell cycle. The aim of Paper III was to study the fragmentation of human embryos and to explore the correlation between fragmentation and the duration of the meiotic and mitotic cell divisions, as assessed by polarized light microscopy of oocytes and time-lapse imaging of embryos. Embryos with a low degree of fragmentation were found to originate from oocytes that had a visible spindle within 35.5 hours, indicating an early completion of the first meiotic division. Time-lapse imaging of embryos revealed that asynchrony between the 2nd and 3rd mitotic divisions was related to a high degree of embryo fragmentation. We suggest that fragmentation of in vitro-derived embryos is related to the progress of the meiotic and the mitotic cell cycles.

1. General introduction

Ovarian ageing as a topic and its associated effects on fertility has received greater attention as an increasing number of women in modern society choose to postpone the age at which they bear children. In Norway, mothers giving birth for the first time had an average age of 28.6 years in 2013, while they were on average 22.9 years old in 1976 [1]. In 2012, for the first time in history, the majority of babies were born to mothers in the age group 30 – 34 years. Until 2012, women in the age group 25 – 29 years delivered most children [1]. Moreover, Northern countries have observed a trend of decreasing fecundity. Indeed, the fecundity in Norway had decreased from 1.98 in 2009 to 1.78 in 2013 [1]. Older women have to face the natural limits of their own reproductive system, due to the fact that fecundity is reduced with increasing female age [2]. Lessons from natural fertility populations show that the fecundity of women decreases gradually throughout her thirties, the decline becoming more significant by 35 of age and accelerating after 40 (Figure 1) [2]. The mean age at menopause is 50-51 years, whereas fertility already terminates ten years earlier, when the woman becomes sterile [2-4]. Observations from reproductive biology, epidemiology, and clinical assisted reproduction support the hypothesis that the time interval between the onset of the accelerated decline of the ovarian reserve and the menopause is more or less fixed. Up to 10% of women in the general population are estimated to become menopausal by the age of 45 and 1% before the age of 40 [5]. These women may have experienced an accelerated decline of their fertility before the age of 32 or even earlier. This has been described as "early ovarian ageing" [4]. Ovarian ageing is characterized by both a reduction in egg quality and a drastic reduction in the total number of ovarian follicles [2].

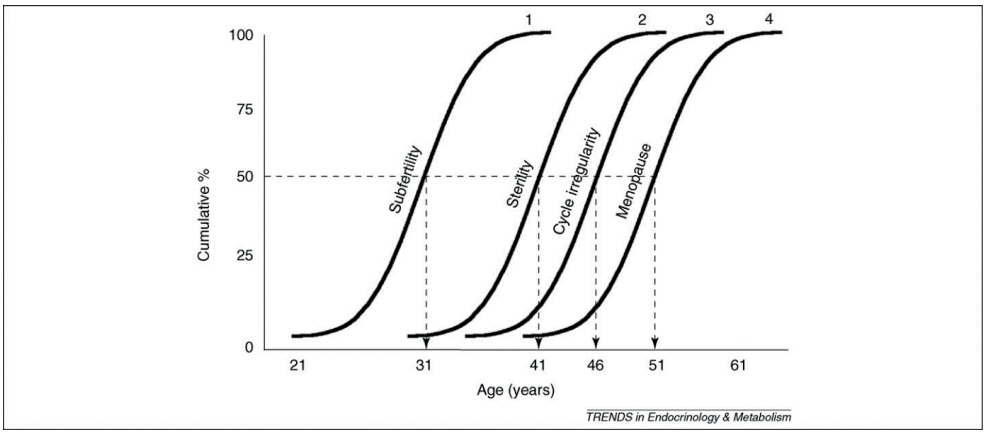

Figure 1: The distributions of age at the onset of subfertility (cumulative curve 1), at occurrence of natural sterility (cumulative curve 2), at transition into cycle irregularity (cumulative curve 3) and at occurrence of menopause (cumulative curve 4). Mean ages for these events are indicated on the x-axis [2].

1.1 Ovarian ageing

In humans, it is firmly established that ovarian ageing progresses constantly during the reproductive lifespan. The relationship between aneuploidy rates in oocytes and increasing age has been established [6, 7]. The reduction of oocyte quality with ageing is believed to be mainly due to an increase in meiotic nondisjunction that leads to an increased rate of aneuploidy in the early embryos [8]. Aneuploidy is the leading cause of reduced fertility in females and developmental abnormalities in fetuses [6, 7, 9]. Indeed, 10-30 % of all oocytes and 50% of the blastocyst stage embryos over all maternal ages may carry a numerical chromosomal aberration [6, 10]. The mechanisms involved in ovarian ageing are incompletely understood, but several hypotheses have been proposed. In sections 1.1.1 – 1.1.7 some prevailing hypotheses are briefly summarized.

1.1.1 Limited oocyte pool hypothesis

Although mitotically active oogonial stem cells have been claimed to be present in the ovaries of mice and humans, it is generally accepted that the population of primordial

follicles is fixed, it is established in the human ovaries during early fetal life, and it serves as the source of developing follicles and oocytes during the entire reproductive lifespan [11-13].
Meiosis I in oocytes is initiated during fetal development, but suspended in diakinesis stage until ovulation, a pause that can last for up to 50 years [14]. The number of oocytes decreases progressively: of 1 million follicles at birth, 450.000 follicles are left at the beginning of puberty and around 1000 oocytes at menopause [2, 4].The "the limited pool" hypothesis postulates that there exists a "production line", so that oocytes that had been formed early during fetal life will ovulate first [15.] According to this hypothesis, the oocyte cohort that enters the growing phase near the end of the reproductive lifespan has a reduced quality, because this late cohort has originated from oogonial stem cells that had undergone more cell divisions and thus may have accumulated more cellular damage [16, 17].
"The limited pool" hypothesis would predict an increased incidence of trisomic pregnancies among women with reduced ovarian reserve independently of age, which has however not been confirmed by epidemiological data. Oocyte aneuploidy that characterizes biological ageing must therefore be determined by factors other than the size of the follicle pool [18].

1.1.2 Two-hit hypothesis of aneuploidy

The current understanding of aneuploidy in oocytes is dominated by the two-hit hypothesis [19, 20]. This model postulates that aneuploidy is a result of two consecutive events that affect the oocyte during fetal life and then during completion of meiosis in oocytes that had already been sensitized by the first event [20, 21]. During the prophase I of meiosis I, homologous chromosomes pair up and form bivalents, which are aligned by the synaptonemal complex [22]. The synaptonemal complex facilitates formation of crossing overs between homologues [23]. Crossing overs are necessary to orient the sister kinetochores towards a single pole at metaphase I [24, 25]. A prolonged lag between entering and completing meiosis may reduce the cohesive ties that hold sister chromatids together and predispose oocytes from older women to aneuploidy, constituting 'the first hit' [26]. The second hit is an inability of the oocyte to detect and respond to recombination failure by activating cell cycle checkpoints, which may deteriorate with age, and thereby allow chromosomes to mis-segregate during meiosis [24, 27].The cell cycle checkpoint may be sensitive to extrinsic,

environmental insults (e.g. smoking, pollutants, etc.), which would accelerate the intrinsic process of ageing accrued through cellular damage, e.g. by reactive oxygen species [21]. Notably, both 'hits' are needed to occur in order to produce mis-segregated chromosomes.

1.1.3 Weakened centromeric cohesion and cohesin loss

The third hypothesis focuses on loading and stabilization of the cohesin complex. During mitosis, a protein complex consisting of four subunits, called cohesin, is required to hold sister chromatids together and allows their bi-orientation on the mitotic spindle; furthermore, cohesin is also important for DNA double strand-break repair and transcriptional control [28]. During meiosis, the sister chromatids are held together from birth until ovulation by cohesin complexes whose ring structure consists of Rec8, SMC1β and SA3 [29, 30]. The maintenance of cohesion between the homolog chromosomes requires preservation of a sufficient amount of cohesin on the sister chromatid centromeres and on the chromosome arms. Gradual decline of cohesin during the long arrest in prophase I may bring cohesin levels to fall below the threshold required to stabilize chiasmata and to hold sister centromeres tightly together, leading to chromosome mis-segregation during metaphase I [31]. Age-related errors of meiosis I may be caused by the inability to regenerate cohesion in oocytes [30]. SMC1β is a meiosis-specific cohesin that allows a controlled release of cohesion during meiosis [32]. Cohesins are regulated by phosphorylation, acetylation, ATP hydrolysis, and site-specific proteolysis, as well as by interaction with cohesin protector proteins, such as Sgo2 [28, 31]. Indeed, cohesion at centromeres is protected by multiple factors including shugoshins (Sgo1 and Sgo2), aurora B kinase, and phosphatase PP2A [33-36]. The shugoshins (Sgos) are conserved centromeric proteins. Sgo1 collaborates with protein phosphatase 2A (PP2A) to protect mitotic cohesin from the prophase dissociation pathway, and Sgo2 is required for the centromeric protection of cohesion in germ cells [34,36]. Aurora B kinase (Aur-B) ensures bi-orientation of sister chromatids by destabilizing incorrectly attached kinetochore microtubules and participates in cytokinesis [34, 37]. The cohesin present on the centromere of sister chromatids has to be protected against separase cleavage in anaphase I in order to prevent premature division of a chromosome into sister chromatids (predivision), which would result in random segregation and aneuploidy [38]. Defects in the kinetochore, in cohesion, or in any of the factors that promote bi-orientation may lead to

chromosome mis-segregation and hence aneuploidy in the daughter cells [39]. Predivision of chromatids is believed to be the major cause of meiotic errors and age-related increase in oocyte aneuploidy (Figure 2) [40].

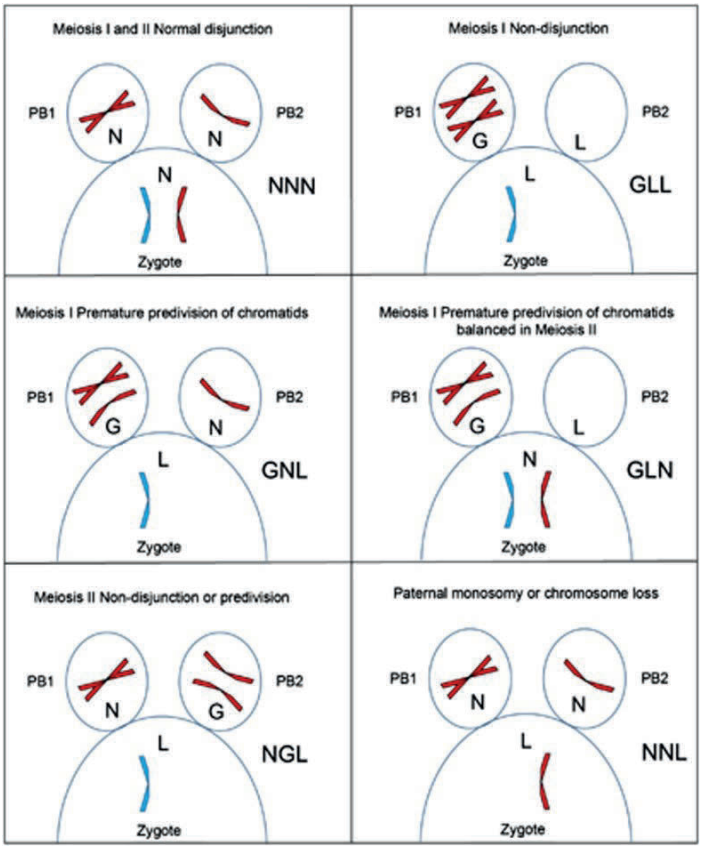

Figure 2: Schematic representation of copy number segregation patterns resulting from normal disjunction, non-disjunction of whole chromosomes and premature separation of chromatids in a mis-segregating chromosome. Maternal chromosomes are presented as red; paternal chromosomes are presented as blue. The segregation pattern is displayed on the right as gain (G), loss (L) or normal (N) copy number for PB1/PB2/Zygote[40].

1.1.4 Function of the spindle assembly checkpoint (SAC) in meiosis

During transition from late prometaphase to metaphase, the spindle assembly checkpoint (SAC) is activated in order to monitor proper attachment of chromosomes to spindle microtubules before anaphase [41-45]. In mitosis, a signal from one unattached kinetochore is sufficient to arrest the cell cycle [46, 47]. Tension across the centromere stabilizes correct attachments, whereas a mechanism involving aurora B kinase corrects the improperly attached chromosomes [48]. In oocytes, the SAC guards against chromosomal mis-segregation but does not induce cell cycle arrest when a few achiasmatic chromosomes (univalents) are present, which may contribute to aneuploidy [49]. Even multiple unaligned kinetochores and severe congression defects can be tolerated by the SAC at the metaphase to anaphase transition, indicating that the checkpoint mechanisms are insufficient in detection or correction of unaligned chromosomes [50]. The age-related reduction in expression of components of the SAC in mammalian oocytes may act synergistically with reduced cohesion between sister chromatids and may predispose oocytes to chromosome segregation errors [51].

1.1.5 Global gene expression and DNA methylation

An alternative hypothesis of ovarian ageing implies global changes in gene expression. During oocyte development, at primordial to primary follicle transition, there is a major change in the global gene expression, followed by a second major change during zygotic genome activation [52]. Indeed, the gene expression profile of the 2-cell embryo differs markedly from oocytes, zygotes, and 8-cell embryos and blastocysts [52]. In oocytes from ageing mice, 5% (530) of the 11,000 detected transcripts showed statistically significant expression changes, including genes involved in mitochondrial function, oxidative stress, chromatin structure, DNA methylation, and genome stability [53].

1.1.6 Shortened telomeres in oocytes

Eukaryotic cells have specialized non-coding DNA sequences at the ends of all chromosomes, called telomeres. The telomeres are protecting chromosome ends from being recognized as

DNA break by nucleases, preventing chromosomal fusions, and properly attaching the chromosome ends to the nuclear envelope. Telomeres in oocytes are believed to shorten with age and, when critically short, contribute to infertility and miscarriage [54, 55]. Telomeres may also be shortened by exposure to reactive oxygen species, so the prolonged interval between fetal oogenesis and ovulation in some women may further contribute to telomere shortening [56]. Telomere shortening in murine oocytes reduces synapsis and chiasmata, increases embryo fragmentation, cell cycle arrest, apoptosis, induces morphological defects of spindle, and chromosome abnormalities. Telomeres are shorter in the oocytes from women undergoing in vitro fertilization, and give increasingly rise to fragmented aneuploid embryos that fail to implant [57].

1.1.7 Advanced glycation end products (AGEs) and the receptor RAGE

A sixth hypothesis of ovarian ageing is the involvement of advanced glycation end products (AGEs), which may contribute to a compromised vascularization and activate an oxidative stress response through interaction with the receptor for advanced glycation end products (RAGE) [58]. Glycation is the non-enzymatic addition of sugars to proteins, lipids and nucleic acids. AGEs are a heterogeneous, complex group of compounds that are formed mainly via the Maillard reaction, which was first described in 1912 by Louis Camille Maillard, a French 16 years old scientist who studied the reaction between amino acids and sugars during heating [59, 60]. The Maillard reaction occurs when a reducing sugar reacts in a non-enzymatic way with amino acids in proteins, with lipids, or with DNA [60]. The formation of AGEs through the Maillard reaction is believed to proceed in three phases. In the first phase, glucose attaches non-enzymatically to a free amino acid, lipid or nucleic acid to form a reversible Schiff base [61]. In the second phase, the Schiff base undergoes chemical rearrangement to form Amadori products, which are more stable than the Schiff base, but the reaction is still reversible [61]. In the third phase, Amadori products accumulate and form crosslinked proteins, which is an irreversible process (Figure 3) [61]. The final brownish products are called advanced glycation end products (AGEs). AGEs may interfere with normal cell function by inactivation of enzymes, abnormalities of nucleic acid function, inhibition of regulatory molecule binding, trapping of proteins by glycosylated extracellular matrix, altered

macromolecular recognition, and increased immunogenicity [61]. AGEs have been associated with several age-related diseases, including diabetes mellitus, cardiovascular diseases, and Alzheimer's disease [62-64].

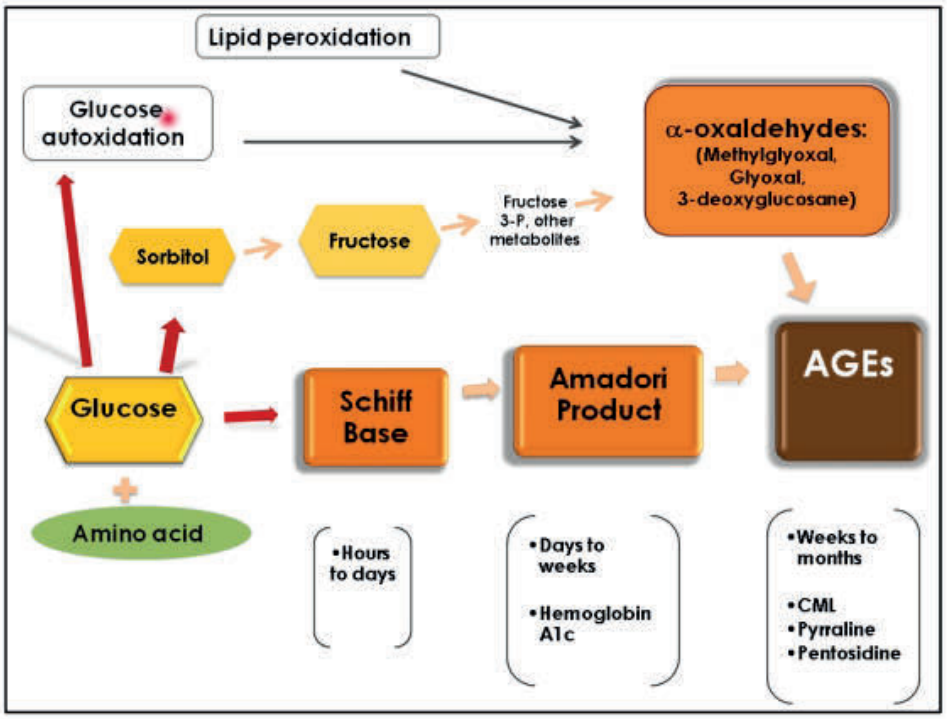

Figure 3: The formation of AGEs occurs in three phases. The results of the first phase are Schiff Bases, the second Amadori products and in the last phase AGEs are produced [65].

The increased concentration of AGEs is believed to influence target cells by three general mechanisms: 1) alteration of function of intracellular proteins; 2) abnormal interaction of extracellular matrix components with other matrix components and with receptors for matrix proteins, such as integrins; 3) induction of receptor-mediated production of reactive oxygen spices (ROS) and cytokines via the AGE receptors (RAGEs) on endothelial cells, mesangial cells, and machrophages (Figure 4) [66].

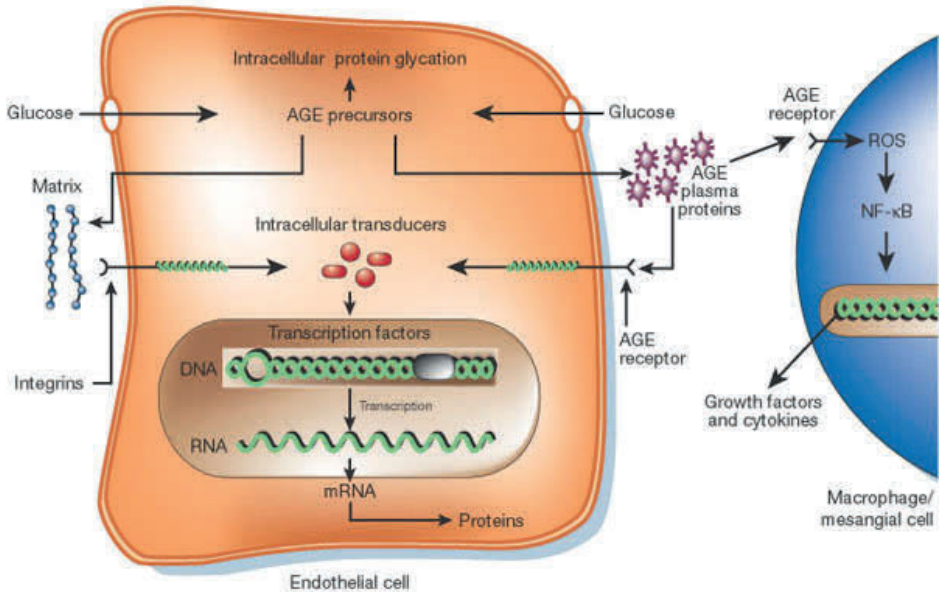

Figure 4: Mechanisms by which intracellular production of advanced glycation end-product (AGE) precursors damages endothelial cells. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGEs creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages [66].

The action of AGEs is classified as receptor independent or receptor dependent [67]. AGEs circulating in the bloodstream may act on cell surface receptors, such as the receptor for AGE (RAGE), which is a member of the immunoglobulin superfamily of receptors[68]. RAGE has transmembrane, cytosolic and extracellular domains [69]. RAGE is abundantly expressed in many tissues, including the heart, lung, skeletal muscle, blood vessel wall, the ovaries, as well as in immune cells, including monocytes, macrophages, and lymphocytes [69]. RAGE activates Nuclear Factor κB (NF-κB) and affects gene expression [66]. RAGE is believed to be down-regulated in most organs in young individuals; with ageing, RAGE expression increases again, possibly due to the accumulation of RAGE ligands, which in turn up-regulate receptor expression [68-70]. Ligand binding induces further up-regulation of RAGE causing a positive feedback loop [68]. In addition to the full-length receptor expressed on the surface of cells, soluble isoforms of RAGE lacking both the cytosolic and the transmembrane domains have been described. These soluble forms of AGE receptors (sRAGE) are secreted by cells and can

be detected in the blood. sRAGE can bind ligands (AGEs) in the circulation, thus preventing the adverse cellular effects of AGE [71].Levels of AGEs in serum and tissues, including the ovary, seem to depend on endogenous and exogenous sources [72]. The endogenous AGEs are produced in the body by chemical reactions. Exogenous sources of AGEs are diet and smoking [73].

The accumulation of AGEs is associated with several reproductive pathologies, including polycystic ovarian syndrome (PCOS) and endometriosis [74-76]. Accumulation of AGEs in tissues induces cellular oxidative stress and promotes inflammation [77]. In the cases of diabetes, inflammation, atherosclerosis, and PCOS, there is a marked induction of RAGE due to the action of its ligands and due to immune mediators derived from activated inflammatory cells [68, 70, 78]. Concentration of circulating AGE derivates, such as carboxymethyl-lysine (CML) and methylglyoxal (MG), positively correlates with indicators of inflammation, such as C-reactive protein (CRP) [79].

1.1.8 Leukocytes in ovarian function

Granulosa cells (GCs) are somatic cells that surround the developing oocyte in the ovarian follicle [80]. The functions of GCs include the production of growth factors that are vital for oocyte development and of sex steroids that regulate ovarian function. Following ovulation, GCs are luteinized and increasingly produce progesterone, which induces secretory transformation of the endometrium and supports pregnancy [81].

Luteinized GCs (GLCs) are frequently used as a model for studying multiple aspects of ovarian function, since these cells can be readily collected by puncture of the ovarian follicle during assisted reproduction procedures. During ovum aspiration, however, the needle penetrates the vaginal wall, ovarian membranes and stroma, and the collected fluid is contaminated with tissue and blood components [82, 83], thus resulting in a heterogeneous cell population in which granulosa lutein cells (GLCs) are mixed with leukocytes [84-86], erythrocytes, as well as other cell types, such as endothelial and epithelial cells. Phenotypic characterization based on histochemical staining or leukocyte-specific immune markers has revealed the presence of macrophages, monocytes, granulocytes and lymphocytes among

these cells [87, 88]. Nonetheless, since both circulating leukocytes (lymphocytes, monocytes, and granulocytes) and tissue-resident immune cells (macrophages/dendritic cells) are observed in the follicular fluid, it has been suggested that these immune cells originate both from a direct bleeding during aspiration and from tissue pieces that are collected in the aspirate [85]. Interaction between ovarian somatic cells and immune cells is thought to be a key regulator of ovarian function. Leukocytes may regulate follicle growth, promote tissue re-organization around ovulation, vascularization and atrophy of corpus luteum. For example, it was shown that ovarian leukocytes increase release of matrix metalloproteinases, chemokines, and angiogenic factors by follicle fluid-derived cells (FFDC) [89, 90]. These interactions are probably mediated by soluble factors, as well as direct cell-cell interactions between leukocytes and ovarian somatic cells. Therefore, careful isolation techniques are necessary if we want to determine the specific role of granulosa cells in follicle development and ovarian function. Studies on luteinizing granulosa cells show that in women aged 38 years and older the granulosa cells are less numerous, produce less steroids and glycoproteins, contain lower levels of normal mitochondrial DNA (mtDNA), damaged mitochondria, and exhibit a reduced expression of antioxidant enzymes compared with GLCs derived from younger women [91-94].

1.2 Assisted reproduction treatment in ageing women

With a rising number of women postponing pregnancy until an advanced age, the demand for assisted reproductive technologies (ART) has increased. The growing popularity of ART has given the impression that female fertility may be manipulated at any stage of life; nonetheless, assisted reproduction technologies cannot reverse the “aged biological clock” of women in the 40’s [95]. Female age is probably the most significant factor influencing clinical outcome in assisted reproduction treatments (ART), and there is a strong association between number of oocytes retrieved and live birth rates [96]. Controlled ovarian hyperstimulation (COH) during ART induces simultaneous development of several follicles. The ovarian response to COH varies substantially among women and is believed to be dependent on the size of the pool of resting follicles, the so-called ovarian reserve, and the responsiveness of the follicles to exogenous stimulation of follicle stimulation hormones

(FSH) [97]. In patients with low ovarian reserve, a reduced follicular response resulting in a low number of retrieved oocytes is achieved even when a high dose of gonadotrophins is administered. Tests for predicting ovarian reserve are available [98]. The incidence of poor ovarian response is estimated to be between 9 – 24 %, values that increase with age and reach about 50% in women older than 40 years [99-102]. The standard definition of poor ovarian response (POR) requires presence of at least two of the following three criteria: (i) advanced maternal age or any other risk factor for POR; (ii) a previous POR; and (iii) an abnormal ovarian reserve test (ORT). Two episodes of POR after maximal stimulation are sufficient to define a patient as poor responder in the absence of advanced maternal age or abnormal ORT [103]. Women who respond poorly to COH have poor pregnancy prospects with a pregnancy rate varying from 7.6% to 17.5% compared with 25.9 – 36.7% in normal responders. Among the prognostic factors within the poor responder group, female age appears to play a distinct role. Overall, older poor responders have lower pregnancy rates (ranging between 1.5 – 12.7%) compared to younger poor responders (13.0 – 35%) [98]. The median of age of women undergoing IVF in United Kingdom is around 36 years and by that time more that 90% of the ovarian reserve has been lost, and the chances of a live birth following IVF are severely reduced [104].

As age increases, the risk of additional disorders that may adversely affect fertility, such as leiomyomas, tubal disease and endometriosis, also increase [105]. Women with a history of prior ovarian surgery, chemotherapy, radiation therapy, severe endometriosis, pelvic infection, or a strong family history of early menopause may be at increased risk of having a premature decrease in the follicular pool and fertility decline. Ageing women more often experience pregnancies with complications, including spontaneous abortion, preeclampsia, gestational diabetes, and placenta previa, and the children of older mothers are increasingly affected by low birth weight, preterm birth, and perinatal mortality [106].

1.3 Embryo assessments in assisted reproduction

Identification of the embryo with the highest potential to implant, establish an ongoing pregnancy and a birth of a child is the assisted reproduction clinic's primary aim. Embryo selection is based on methods that can give a direct or indirect clue regarding the potential

of a given embryo to implant. These methodologies are based on either invasive or noninvasive procedures, whereas the noninvasive procedures, described briefly in sections 1.3.1 – 1.3.3, are routinely applied in assisted reproduction laboratories.

1.3.1 Morphological embryo assessments

In most IVF laboratories, embryo selection is based on morphological evaluation using light microscopy. There have been some concerns about the detrimental effects of light exposure on embryos or on cell culture media, but several publications reported no detrimental effect on embryo development of light exposure on mouse oocytes, rabbit embryos and human oocytes [107-110]. Visual observations are typically carried out at three distinct developmental stages: fertilization, cleavage stage, and blastocyst stage. The morphological criteria considered include: number of pronuclei and polar bodies at fertilization (zygotes); cell number, degree of cellular fragmentation, evenness of mitotic divisions, presence of multinucleated blastomeres (cleavage stage embryos); extent of blastocoel expansion and assessments of inner cell mass (ICM) and trophectoderm (TE) (blastocysts) [111-115]. Important considerations during morphological assessments of embryos include: 1) reducing the disturbance of embryo culture as much as possible (which may result in assessing embryos only once per day), 2) observing embryos at developmentally relevant time points.

Morphological characteristics of gametes and embryos may predict implantation, pregnancy, and live birth [116].The rate of which the embryo development proceeds is believed to be of great importance, and transferred embryos that have four cells on day 2 and eight cells on day three post-insemination are expected to have a maximal implantation rate [117-119]. Embryos that have cleaved more slowly than the expected rate have a reduced implantation potential, and embryos that have cleaved faster than the expected rate are likely to be chromosomally abnormal and also have a reduced implantation potential [117].

Early cleavage is defined as a division to a two-cell embryo within 25- 27 hours post-insemination, and its prognostic impact was first described by Edwards in 1984 [120]. Early cleavage is believed to correlate with increased pregnancy rates [121, 122].

Multinucleation is defined as the presence of more than one nucleus in a blastomere, including micronuclei, and is associated with chromosomal abnormalities, impaired developmental capacity, decreased implantation potential, and an increased risk of spontaneous abortion [115, 117, 123]. In a recent study, the proportion of mononucleated blastomeres had a strong predicting power for live birth [119]. Nonetheless, embryos with a binucleated blastomere at two cell stage can develop further, suggesting that embryos may correct multinucleation [124].

Cleavage of embryos resulting in blastomeres with uneven size is supposed to negatively affect the developmental capacity of the embryo, lower pregnancy and implantation rates, and has been associated with multinucleation and aneuploidy [125, 126].

A fragment is defined as an extracellular membrane-bound cytoplasmic structure that is < 45 µm diameter in a day-2 embryo and < 40 µm diameter in a day-3 embryo [127]. Fragmentation of human cleavage-stage embryos is a dynamic process with appearance of fragments and absorption in blastomeres [128]. Fragments may also be positioned between blastomeres or along cell division planes and interfere with compaction, cavitation, and blastocyst formation [129, 130]. It has been demonstrated that fragments may contain even whole chromosomes, suggesting that fragments may contribute to aneuploidy in mosaic embryos [131]. Embryo fragmentation in cleavage-stage embryos is found to be directly proportional to chromosomal abnormality [132, 133]. Comparative genomic hybridization with DNA microarray revealed that high proportions of human blastocysts from women of advanced maternal age are aneuploid or mosaic [134]. Indeed, fragmentation may qualify as an independent predictor of live birth, and only marginally less strong predictor than cleavage rate, and comparable to the presence of mononucleated blastomeres [119].

1.3.2 Assessments of the meiotic spindle

Visualization of the meiotic spindle by polarized light microscopy is a non-invasive tool to assess the developmental status of the oocyte. In life sciences, polarized light microscopy was used to visualize sperm as early as 1875, sea urchin embryos in 1937, and spindle

dynamics from the 1950s - 1970s [135]. As technology has advanced and the technique become more sensitive and compatible with live cells and real-time imaging, it became available for routine examination of gametes and embryos [136-138]. Polarized light microscopy instruments include an inverted light microscope fitted with a polarizer, a rotating liquid crystal retarder, a camera, and computer software to analyze the digital images [136]. This technology uses polarized light to illuminate birefringent structures, which are highly ordered molecules such as microtubules in spindles or glycoproteins in zona pellucida (Figure 4) [139].

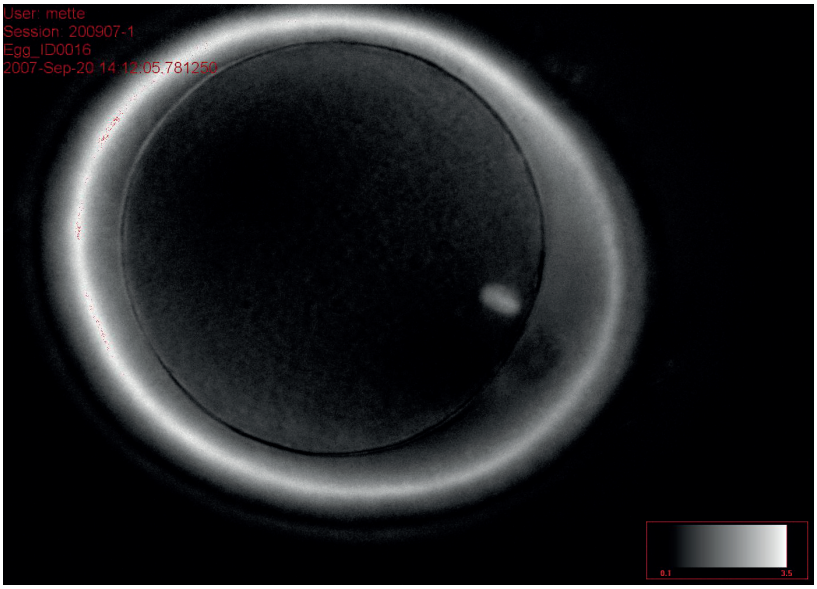

Figure 4: Meiotic spindle and zona pellucida as seen by polarized microscopy, Polscope.

The birefringent structures are visualized as bright objects, and the shift of wavelength of light while passing through the structure is measured as retardance (nm) [139]. Presence of birefringent spindles in human oocytes is suggested to predict higher fertilization and embryo development rates [139-144]. Although the clinical use of polarized microscopy is debated, the visualization of the meiotic spindle may provide insight into oocyte maturational or possibly chromosomal status [145, 146]. It is important to note that the meiotic

spindle is a dynamic structure, and it is affected by environmental conditions, such as temperature [141, 147].

Completion of the first meiotic division is marked by the formation of the first polar body (PB1), and failure to proceed to first polar body formation is termed meiosis I arrest. Metaphase I (MI) arrest is believed to occur because of a defect in the signal transduction pathway mediating meiotic progression or spindle abnormalities, which may result in activation of the spindle assembly checkpoint (SAC)[148]. Progression through meiosis I to metaphase II arrest requires chromosome condensation, formation of the metaphase spindle, chromosome alignment along the first metaphase plate, followed by the separation and segregation of homologous chromosomes.

Metaphase II (MII) oocytes are generally considered as "mature" and are presumed to be capable to fertilization. Arrest at metaphase II is initiated and maintained by Emi2, the MOS/MAPK pathway, and SAC [149]. Oocytes deficient in Emi2 were not able to form a spindle complex during meiosis II and were arrested at MII [150].

1.3.3 Morphokinetic assessments of embryos

In contrast to usual daily assessment that gives a snapshot of the dynamic process of embryo development, with time-lapse technology, embryos can be monitored without removing from the incubator and thereby maintaining optimal culture conditions. Several groups have affirmed that fertilization, embryo development, blastocyst formation and implantation potential of embryos cultures in time-lapse instruments is comparable to standard incubators [151-154]. One of the first time-lapse studies of human embryo development was conducted in 1997 [109]. Since then, many papers have been published concerning the subject of time lapse imaging of human embryo development, especially morphokinetic assessment, which combines morphology with the timing of cell divisions. Nonetheless, there has been no consensus on whether time-lapse technology was improving live birth rates, safety, and cost effectiveness [155]. Indeed, a recent Cochrane review concluded that there is insufficient evidence for a superior clinical outcome with time-lapse incubators over standard incubators

[156]. Furthermore, the utility of time-lapse technology to predict aneuploidy in human embryos has been highly debated [157-160].

Nevertheless, time-lapse technology is a superior tool for studying the dynamic process of embryonic growth, as static observations are incapable of assessing progressive events and some important phenomena may be missed or overlooked [161]. For example, it has been described that timing and synchrony of the first cleavage divisions is critical for normal development. In addition, traditional snapshots at specific developmental stages are unreliable to detect dynamic processes like multinucleation, emergence and disappearance of fragments, and collapse and re-expansion of blastocysts.

2. Aims of the thesis

The aim of this thesis was to explore and evaluate clinical and molecular derangements associated with female reproductive ageing during assisted reproduction treatment.

Specific objectives were:

1) Examine whether morphological scoring systems routinely used in assisted reproduction laboratories reflect the female age-related impairment of oocyte and embryo quality.
2) Explore whether the advanced glycation end products (AGEs) and the receptor (RAGE), known to be involved in other ageing-related diseases, are expressed on the surface of follicle fluid-derived cells.
3) Study the process of fragmentation of human embryos and examine whether it was related to the progression through meiotic and mitotic cell cycles.

Additional aims were:

4) Assess whether visualization of the meiotic spindle was related to female age.
5) Assess whether morphokinetics of the embryo development was related to female age.

3. Methods

3.1 Study design

In this thesis we aimed to explore the impact of female reproductive ageing on assisted reproduction treatment, specifically the effects on embryo development and on ovarian cells *in vitro*. In Paper I, we aimed to identify clinical parameters that were associated with ageing in a retrospective epidemiological study using a large clinical database. In Papers II and III, we tested specific hypotheses on the effect of ageing on ovarian cells and on embryo developmental kinetics, and conducted prospective studies using isolated follicle fluid-derived cells, oocytes, and embryos.

3.2 Methods

3.2.1 Ovarian stimulation and oocyte retrieval

Ovarian stimulation was performed according to the mid-luteal phase down-regulation protocol. Final follicular maturation was induced with human chorionic gonadotrophin (hCG), and ultrasound-guided transvaginal oocyte retrieval was scheduled 34 – 38 hours later. The follicle fluid was searched for oocytes under a microscope, and the oocytes were placed in IVF media. In Paper II, the follicular fluid was further processed to isolate follicle fluid-derived cells.

3.2.2 Gamete assessments and ICSI/IVF procedure

Intracytoplasmic sperm injection (ICSI) was indicated in case of male factor infertility, reduced sperm quality on the day of oocyte retrieval, or history of reduced fertilization with in vitro fertilization (IVF). Prior to ICSI, the cumulus cells surrounding the oocytes were gently removed and the oocytes were classified according to maturity as germinal vesicle (GV), metaphase I (MI), or metaphase II (MII). Prior to IVF, the maturity of the cumulus-oocyte-complex was evaluated by the appearance of the cumulus mass. Semen samples were subjected to either swim-up preparation, gradient centrifugation, or washed with sperm

preparation medium. IVF was performed by addition of approximately 100 000 motile spermatozoon to each oocyte. ICSI was performed on metaphase II oocytes only.

3.2.3 Meiotic spindle observation

In Paper III, the oocytes were subjected to polarized light microscopy with the LC-Polscope system to visualize the meiotic spindle. Observations were performed 35.5 – 40.5 hours after hCG injection on a heated stage to reduce temperature fluctuations. The appearance of a spindle and the angle respective from the first polar body were denoted. The image analysis was performed by a single trained observer.

3.2.4 Morphological assessments of embryo

Normal fertilization was determined by appearance of two pronuclei (PN) and two polar bodies. In Paper I, the fertilization was assessed 18 – 19 hours post-insemination. In Paper III, the oocytes that had been imaged by the Polscope were checked for fertilization 19-20 hours post injection, whereas in case of the oocytes cultured in the EmbryoScope, the exact time point when the two pronuclei were seen was recorded.

In Paper I, the embryo quality was assessed on day 2 and day 3 after oocyte retrieval (44 – 45 h and 68 – 69 h post-insemination). The blastomeres were counted and embryos were given a blastomere score, where the top score was given to a 4-cell embryo on day 2 and an 8-cell embryo on day 3. Embryos with fewer or more blastomeres received a lower blastomere score. The embryos were also given a morphological score based on embryo fragmentation and the blastomere size (even/uneven sized blastomeres). Embryos with no fragmentation and equally sized blastomeres achieved the highest morphological score. The total embryo score was based on the blastomere score and the morphology score. Top-quality embryos with both a maximum blastomere score and a maximum morphology score were given the maximal total embryo score. Since transferring two embryos may increase the possibility of a pregnancy compared to transferring of a single embryo, the sum of scores was also calculated.

3.2.5 Morphokinetic assessments of embryos

In Paper III, the microinjected oocytes were subjected to time-lapse recording in the EmbryoScope. Images were acquired every 20 minutes in seven focal planes, and both morphological assessments (fragmentation, number of cells, size of blastomeres, number of nuclei, multinucleation) and key cytokinetic events were evaluated and marked by a single trained observer. The cytokinetic events denoted were: second polar body extrusion, fertilization, the first mitotic division (t2), second mitosis (t3), third mitosis (t4). The degree of fragmentation was recorded as it evolved during embryo development and at the time-point before transfer.

3.2.6 Studies on isolated ovarian cells and leukocytes *in vitro*

Human follicle fluid-derived cells (FFDC) were isolated from follicle aspirates using hemolysis, enzymatic and mechanical dispersion, and gradient centrifugation. Peripheral blood-derived mononuclear cells (PBMC) were isolated by standard procedures.

For immunofluorescence microscopy, cells were cultured on glass coverslips, fixed with paraformaldehyde, permeabilized, and labelled with primary and secondary antibodies.

For flow cytometry, cells were either fixed in paraformadehyde or the Cytofix/Cytoperm reagent, followed by exposure to primary and secondary antibodies. Analysis was performed with a FACScan instrument (BD) using the Cellquest 4.0 software (BD) or FlowJo. Propidium iodide (PI) was added immediately before analysis to identify and exclude necrotic cells.

Binding and uptake of fluorochrome-conjugated bovine serum albumin (BSA) and BSA conjugated with advanced glycation end products (AGE-BSA) by cultured FFDC was examined with immunofluorescence microscopy and quantitated by flow cytometry. To affirm a specific binding of AGE-BSA on the cell surface, the cells were incubated with mannan, D-mannose or D-galactose as compatitve binding inhibitors. Activation of apoptosis-associated signaling pathways and cell death in FFDC was examined with immunofluorescent microscopy and flow cytometry. These experiments included: detection of phospho-NF-κB p65 in cells, which would indicate activation of the NF-κB pathway; detection of cleaved caspase-3, a key effector of the cell death pathway; binding of annexin V by cells, which

indicates phosphatyldilserine switch in the cell membrane during the initial steps of apoptosis; detachment of cells during monolayer culture, indicating cell death.

3.3 Statistical analysis

In Paper I, association between age and multiple outcome variables was assessed with analysis of variance or chi-squared test for trends. The effect of age and treatment characteristics on pregnancy odds were analyzed with logistic regression, using univariate and multivariate models to assess the effect of a variable alone and after adjusting for covariates.

In Paper II, Pearson correlation, Student`s t test and analysis of variance were calculated as appropriate.

In Paper III, non-linear regression analysis was used to correlate the appearance of the meiotic spindle to the degree of fragmentation, and ANOVA for linear trend was used to compare the time-points for emergence of pronuclei and mitotic progression of cell cycles to fragmentation.

4. Summary of results

Paper I

Advanced chronological age was found to be associated with fewer oocytes retrieved, fewer embryos available for cryopreservation, as well as lower pregnancy, implantation, live birth rates, and a higher miscarriage rate. No age-related correlation was found between fertilization rates, oocyte or embryo quality assessed as blastomere score, morphology score, and the total embryo score of transferred embryos.

Paper II

GL cells and ovarian monocytes were found to express both AGE and RAGE on the cell surface. RAGE-specific fluorescence intensity in GL cells correlated with the patients' chronological age. GL cells, monocytes, and lymphocytes were found to bind AGE-albumin (BSA), and the binding of AGE-BSA of GL cells correlated with the patients' chronological age. Binding of recombinant human chorionic gonadotrophin to GL cells was not affected by the presence of AGE-BSA. In GL cells, AGE-BSA and BSA failed to induce cleavage of caspase-3, phosphorylation of NF-κB, and increased binding of annexin V. AGE-fibronectin was found to induce detachment of cultured GL cells *in vitro*. The findings support that ovarian granulosa cells and monocytes are exposed to AGEs *in vivo*, express RAGE, and bind AGEs on the cell surface. The ligands of RAGE and their effects in the ovary remain uncertain. Structural long-lived ECM proteins rather than soluble AGEs may play a role in the decline of ovarian function during ageing.

Paper III

Human embryos with low degree of fragmentation (< 10%) at 42- 45 hours after insemination, originated from oocytes with an early appearance of the meiotic spindle (35.5.hours after hCG injection), early first mitosis (27.7 hours after insemination), late start of the second mitosis (38.5 hours after insemination), and a shorter duration of the third mitosis (0.6 hours). Conversely, highly fragmented embryos (> 50 % fragmentation)

originated from oocytes with a late appeared meiotic spindle (36.5 hours after hCG injection), late start of the first mitosis (28.7 hours after insemination), early start of the second mitosis (37.6 hours after insemination), and a longer duration of the third mitotic cell cycle (2.2 hours).

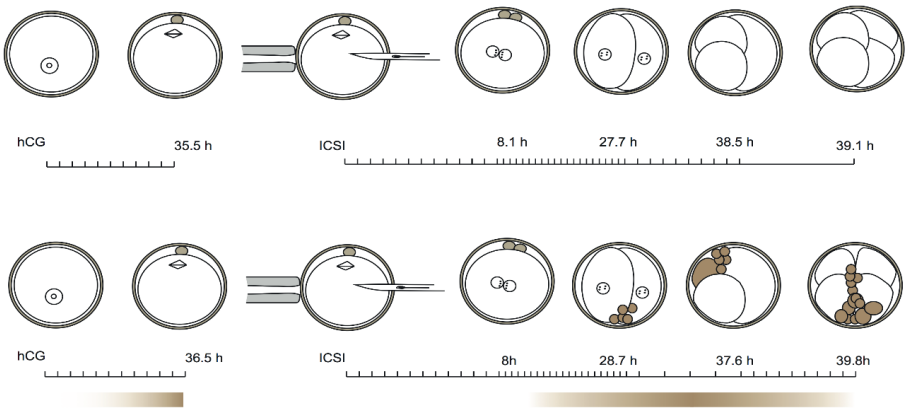

Figure 5: Summary of the results presented in Paper III.

5. General discussion

This thesis examined the effect of age on treatment outcome among women undergoing assisted reproduction treatment, including morphological assessment of oocytes and embryos, visualization of the meiotic spindle, and morphokinetic analysis in a time-lapse system. Furthermore, this thesis examined a specific mechanism of ageing, the AGE-RAGE system, in cells derived from the ovarian follicle. In Paper I, we found that morphological assessments, such as degree of fragmentation, fail to detect age-related impairment in oocytes and embryos. This result made us interested in the process and development of embryo fragmentation, which was the subject of Paper III. In section 5.2.3 we report additional analyses that examined the association between ageing, spindle emergence, and embryo fragmentation.

5.1 Methodological considerations

5.1.1 Paper I

Paper I is a retrospective study of 4587 women undergoing their first assisted reproduction treatment during 1996 – 2007. The paper revealed that morphological assessment done routinely at assisted reproduction clinics fails to reflect age-related changes in oocytes and embryos. The strength of this paper is that over 43,000 oocytes, 25,000 embryos and 7900 transferred embryos are assessed. Some limitations are its retrospective nature and that patient records were collected over a long period of time. Although a single embryo scoring system was used throughout Paper I, many embryologists have contributed to the assessments of oocyte and embryo quality in this paper, and it is known that morphological assessments are prone to subjectivity [162].

Standard scoring of cleavage-stage embryos using microscopic evaluation by an embryologist has some notable limitations. Firstly, lack of a clearly defined standard method to measure and record specific characteristics can lead to loss of important information, and different laboratories often assess oocytes and embryos with different scoring systems. However, none of these grading systems has been properly validated in terms of reliability.

Secondly, as morphology and developmental competence are not firmly correlated, morphological assessment is known to have limited predictive value in the identification of the most viable embryos [163]. A newly published study examined 1213 embryos by comprehensive chromosome analysis and revealed that chromosomal abnormalities were common among the cleavage stage embryos that had been assigned the best morphological scores [164]. However, at blastocyst stage aneuploidies were found to be significantly less common among top quality blastocysts compared to blastocysts with poor morphology [164]. Some variation in scoring of morphological parameters can also be expected among different embryologists (inter-observer variability) and within the same embryologist (intra-observer variability) [165]. However, many studies affirmed an acceptable intra-observer and inter-observer variability during the evaluation of embryo morphology [162, 166, 167].

Thirdly, due to the workload of the laboratory and the importance of a stable environment for the oocytes and the embryos, the morphological assessment may not always be done at constant time points and embryos may be scored once or twice a day. Fluctuations in pH and temperature may have deleterious effects on oocyte and embryo development [168]. Since a rapid evaluation is therefore essential, important events may be missed or overlooked, especially dynamic processes such as emergence of cellular fragments and multinucleation of blastomeres.

Since the predictive value of oocyte morphology is controversial [169-175], we concentrated on assessing the maturity of oocytes. Oocytes fertilized by ICSI were evaluated with the cumulus cells removed during the denudation process, whereas oocytes fertilized with the IVF were evaluated by assessments of the cumulus-oocyte complex [176], although this latter method may be inaccurate predicting oocyte maturity [177, 178]. The embryos were evaluated on day 2 and day 3 after ovum pick up for morphological assessment, and were given a blastomere score based on the number of blastomeres and a morphology score based on the degree of fragmentation and size of the blastomeres. The number of blastomeres has been proposed to be the key morphological factor to predict pregnancy and live birth, and the ideal cleavage rate on day 2 and day 3 to be four and eight blastomeres, respectively [111, 114, 179, 180]. Therefore, the maximum blastomere score was given to embryos with four blastomeres on day 2, and embryos with more or less blastomeres were given lower scores. Calculating the blastomere score, there were corrected for abnormal features in the

blastomeres (vacuoles, centralized cytoplasm, granularity, vacuoles, symmetry, abnormal shape of the blastomeres etc). Each abnormal feature contributed with a minus point to that certain embryo's blastomere score.

Degree of fragmentation and size of blastomeres were defining features of the morphological score (Table 1). It is well established that fragmentation is related to lower implantation and pregnancy rates and may be indicative of chromosomal abnormalities [118, 120, 129, 132, 133, 181, 182.] The presence of uneven sized blastomeres has been shown to be associated with inferior pregnancy and implantation rates and may be related to aneuploidy [118, 125, 180, 183, 184].

Table 1: Morphological embryo scoring based on fragmentation and blastomere size.

Score	*Description*
4	Equally sized blastomeres and no fragmentation
3	⩽10–20% fragmentation, even or uneven blastomeres
2	>20–50% fragmentation, even or uneven blastomeres
1	Fragmentation precluded counting blastomeres
0	Cleavage arrest or morphologically abnormal embryo

5.1.2 Paper II

In Paper II, we cultured follicle-fluid derived cells isolated from aspirates of ovarian follicles from women who underwent assisted reproduction treatment. Since the experiments required large numbers of cells, fluids of multiple follicles were pooled, which may imply some disadvantages. Firstly, revealing the oocyte and embryo quality of each follicle would have been an apprehensive addition to the status of the follicle fluid-derived cells. Secondly, number of follicle fluid-derived cells did correlate with the number of follicles/oocytes. Women at advanced age, even when the follicles were pooled, had fewer follicles to aspirate and therefore fewer cells to be analyzed with the flow cytometer.

Immunofluorescence microscopy was used to demonstrate the presence and distribution of AGE and RAGE through the cells. For quantitative analysis, flow cytometry was used. We aimed to study the effect of AGE on follicle fluid-derived cells, especially the granulosa lutein cells (GLC). One obstacle in studying these cells with flow cytometry is that there exists no single cell surface antigen, or a combination of multiple markers, that would readily distinguish granulosa lutein cells. Instead, we negatively selected contaminating immune cells expressing common immune cell markers. Optimally, immune cells could have been excluded with fluorescence-activated cell sorting (FACS), a specialized method with which cell populations expressing a specific marker can be sorted.

The isolated granulosa lutein cells were cultured on gelatin-coated dishes *in vitro* to study the intracellular effects of AGE and RAGE activation. The plating density of cells may be an important factor due to the differentiation of the granulosa lutein cells. One study has reported that a lower plating density could make granulosa cells exhibit estrogen production, while at a higher plating density the granulosa cells may appear as progesterone producing theca lutein cells [185]. It is therefore uncertain whether this may be one obstacle with Paper II, due to the fact that we cultured various number of cells per volume of culture media depending on the number of follicles aspirated.

The intracellular response of AGE, binding of AGE to RAGE, and activation of apoptosis were studied in Paper II. Intracellular cascades of apoptosis and intracellular responses to RAGE activation are numerous. Annexin V was selected as an early marker of apoptosis due to the fact that apoptotic cell death is accompanied by loss of phospholipid asymmetry in membrane structures and surfacing of phosphotidylserine (PS) on the outer membrane leaflet [186]. Annexin V positive granulosa lutein cells have been found by others [187, 188].

Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Activation of caspase-3 was examined in Paper II, as it is an effective readout of apoptosis in other systems, like neuronal death in Alzheimer's disease [189, 190].

Oxidative stress in cultured luteal cells was shown to induce apoptosis through the activation of NF-κB [191]. Due to the fact that, in Alzheimer disease, NF-κB activation is induced by activation of RAGE [192], NF-κB was selected as readout in Paper II.

5.1.3 Paper III

In Paper III, we used polarized light microscopy to examine the meiotic spindle, which has birefringent features. Birefringence is an optical property of highly ordered molecules such as microtubules in spindles and oriented glycoproteins in the zona pellucida. The birefringent structures are seen as bright structures in a polarized microscope [139]. The visualization of the meiotic spindle as a marker of oocyte quality is controversial [193].

Although the polarized light may be harmless to the oocytes, some temperature fluctuations may affected our observations, despite the fact that we maintained constant temperature by a heated stage [144]. The optimal method of visualization of the meiotic spindle would have been a time lapse system to study the dynamic nature of the spindle, since meiotic spindles disappear and reappear during oocyte maturation [141].

One obstacle of this study was that there were many embryologists involved in visualization of the spindle, but a single trained embryologist analyzed the images.

In one prior article, the authors found no consistent correlation between morphometric evaluations of the spindle through Polscope with confocal analysis by immunostaining of the microtubules, and the Polscope was suggested to be an inefficient method for assessing the metaphase II spindle and for noninvasive oocyte selection [194].

Since the first time-lapse study in 1997 by Payne and colleagues [109], morphokinetics has added time-lapse evaluations to regular morphology evaluations. Nonetheless, a recent Cochrane report concluded that there is insufficient evidence for improved live birth, miscarriage, stillbirth or clinical pregnancy rates in time-lapse systems compared to standard incubation [156]. Many of the time-lapse studies published are retrospective and the risk of bias is strong and difficult to account for. Nevertheless, there are several practical advantages of time-lapse systems. Firstly, time-lapse systems provide strictly controlled and stable incubation conditions that may be an improvement over regular incubators [151, 153].

Secondly, minimal handling of oocytes and embryos inside and outside of the incubator is advantageous. The only time-points the embryos are outside the incubator are when loading of the oocytes in the EmbryoSlide and during changes of the media at culturing to blastocyst stage. Thirdly, time-lapse systems provide more information of the embryo development for qualitative evaluation of morphology, especially dynamic processes such as fragmentation and multinucleation. Time-lapse monitoring gives the chance to observe the embryo continuously and enables the embryologists to detect any changes that occur during embryo development. And lastly, quantitative morphokinetic parameters allow selection of viable embryos. As transfer of blastocysts with deviant morphokinetic parameters may result in live births [195], a morphokinetic model should be used for ranking rather than selection to avoid the risk of discarding usable embryos [196].

For studying the dynamic processes of embryo development, such as cytokinesis, the appropriate interval between the images should be defined. Time-lapse systems are normally programmed to 20 minutes intervals. To be able to cover rapid processes, it should be programmed to 5 min intervals, which several of the various time-lapse systems are capable of. Wong et al. predicted the progression to the blastocyst stage with >93% sensitivity and specificity by measuring three dynamic, noninvasive imaging parameters by day 2 after fertilization, before embryonic genome activation (EGA) [131]. Problems during cytokinesis, which may cause longer cell cycles, may be both cause and consequence of chromosome abnormalities [197].

One obstacle with time-lapse recordings is that start and termination of dynamic events may be difficult to define. For example, a specific dynamic event may be regarded to start at its initiation, e.g. the first frame of observation, or alternatively, when the feature achieved its maximum. Even though the intra-observer and inter-observer consistency of time-lapse annotation was shown to be high [198], each laboratory is advised to specify its own morphokinetic parameters and to adopt a uniform approach to time-lapse terminology and definitions of morphokinetic variables to evolve improved interpretation of time-lapse data [199].

Additional limitations with the time-lapse systems are that 1) embryos are non-adherent and may move out of the field of view of the microscope, 2) formation of air bubbles may prevent embryo evaluation, 3) difficulties in assessments of number of cells in advanced cleavage or blastocyst-stage embryos, and finally 4) assessing the degree of fragmentation in high fragmented embryos. Even though the time-lapse system has the possibility to view an embryo through different planes, the embryos cannot be rotated.

The concerns about the prolonged exposure to light in time-lapse systems is likely to be unfounded. The different systems use red light, which is believed to be less disturbing for the embryo development. One animal study found no significant difference in blastocyst development in embryos expelled to red light compared to embryos viewed by a regular inverted microscope [200].

5.2 Interpretation of results

5.2.1 Interpretation of results - ageing and assisted reproduction treatment

In Paper I, we found that even though younger women received more prolonged stimulation with follicle stimulation hormone (FSH) compared to older women, older women received a significant higher dose of total FSH. Despite the higher dose of follicle stimulating hormone, reduced numbers of oocytes were retrieved, confirming earlier data [201-203]. Even though the number of oocytes was lower compared to young patients, the quality of oocytes as assessed by oocyte maturity was comparable and the fertilization rate were not different between the age groups, in agreement with previous reports [203].

In line with others, we also failed to find age-related trends affecting embryo development, assessed by the mean morphology score, the mean blastomere scores, and the cumulative total score of transferred embryos [204-207]. Nonetheless, the association between maternal age and embryo quality has been controversial. It has been suggested that maternal age does not affect the relationship between euploidy and day-3 morphology [208]. Another publication reported significantly reduced cleavage in embryos from women of advanced age [209]. Ziebe and colleagues found no age-related decline in number of blastomeres, but described an age-related increase in proportion of fragmentation [201].

We found that pregnancy rate, implantation rate and live birth rate declined with increasing age, and the miscarriage rate increased, in agreement with previous reports [203, 210]. To verify that the oocyte and embryo quality scores were correlated with pregnancy, we analyzed the effect of age and treatment characteristics on pregnancy odds with logistic regression. Both univariate and multivariate analysis gave the similar result and confirmed that age reduces the odds for pregnancy, and that the number of oocytes retrieved, oocyte quality score, embryo morphology score, embryo blastomere score and cumulative total embryo score of transferred embryos contributed to increased odds for pregnancy.

The results in Paper I may justify the practice of transferring two embryos in women with advanced age, even though this might increase the possibility of multiple pregnancies. One may still need to develop non-invasive methods to detect age-related impact on gametes and embryo development routinely in the laboratories.

5.2.2 Interpretation of results - ageing and AGEs in the ovary

We confirmed using immunofluorescence and flow cytometry the expression of AGE and RAGE on the surface of follicle-fluid derived cells. Expression of RAGE and binding of AGE-BSA was found to correlate with the patients' chronological age. AGE-BSA was highly bound by GL cells. A potential accumulation of AGEs in the human ovary may account for a number of age-related features of ovarian dysfunction, including impaired vascularization and consequent hypoxia and reduced intake of nutrients by follicle cells [58]. A possible correlation of AGE with follicle ageing was first reported by an observational study that found increased levels of pentosidine, an AGE compound, in the primordial, primary, and atretic follicles of premenopausal women [211]. Another study provided evidence for a potential role of AGE signaling in the control of the ovarian extracellular matrix during follicular development [212], implicating inflammatory mechanisms in ovarian dysfunction during ageing.

Elevated expression of AGE and RAGE has been found in other reproductive diseases connected to inflammation. Studies by Diamanti-Kandarakis and colleagues affirmed the presence of AGEs in ovarian tissue of young women and also described increased levels in serum and ovary of women with polycystic ovary syndrome (PCOS) [74, 213]. The serum levels of the soluble form of RAGE, lacking both the cytosolic and the transmembrane domains, has

been described to be elevated in patients with polycystic ovarian syndrome (PCOS) [214]. Other groups examined the role of AGE and RAGE in patients with endometriosis and found elevated expression of RAGE in endometriosis compared to healthy endometrial tissue [215]. Increased serum levels of sRAGE of women with recurrent pregnancy loss (RPL) may indicate that AGE-RAGE is involved in RPL [216]. It has been reported that maternal sRAGE levels are higher in patients with preeclampsia (PE) than in women with normal pregnancies [217, 218], further supporting the pathological role of AGE via RAGE, probably involving inflammation.

There have been several reports connecting AGE-RAGE to outcome of assisted reproduction treatments. Levels of sRAGE in the follicle fluid of patients undergoing assisted reproduction treatments have been found to correlate negatively with the total gonadotropin dosage needed per cycle, independently of age, BMI, or day 3 serum FSH level. Follicular fluid sRAGE levels were also suggested to predict the number of oocytes retrieved [219]. This latter study also reported that concentration of sRAGE in the follicular fluid was several-fold higher compared to serum and most other biological fluids, suggesting a preferential accumulation of sRAGE in the follicular environment [220]. In addition, it was reported that serum sRAGE correlates negatively with the number of follicles and oocytes, together with high follicular sRAGE levels, in particular in women who conceive. Indeed, plasma concentration of sRAGE was found to be significantly higher among young women, and the level of sRAGE found in the follicle fluid showed a tendency of positive correlation with the number of oocytes retrieved [221]. A positive correlation between levels of sRAGE in follicular fluid and follicular fluid levels of anti-Müllerian hormone (AMH) was also described [219]. Jinno et al. [222] found that accumulation of the so-called toxic AGE (TAGE) in follicular fluid and in serum correlated negatively with follicular growth, fertilization, and embryonic development. TAGE correlated positively with altered glucose metabolism, age, and factors related to obesity, dyslipidemia, hyperglycemia, and insulin resistance.

In Paper II, we found that exposure of cells to AGE-BSA up to a concentration of 200 µg/ml failed to activate markers of apoptosis, such as annexin V, caspase -3 and NF-κB. Reber and colleagues reported that exposure of neural cells and glial cells to 500 nM AGE-BSA was not followed by apoptotic or necrotic cell death [190]. In ovarian granulosa-lutein cells, activation of NF-κB and caspase-3 was associated with a high rate of apoptosis, in particular during the late luteal phase [191]. In neurons, it has been shown that methylglyoxal, a precursor of

advanced end glycations, triggers apoptosis by inducing activation of caspase-3, suggesting a role of AGEs in neurodegeneration in Alzheimer`s disease [189]. We found that AGE-fibronectin induced follicular-fluid derived cell death, which may imply that, rather than soluble AGE, modification of structural long-lived extracellular matrix proteins by AGE may be a culprit in the ovarian dysfunction of ageing.

Notably, by using staurosporine, which is a known inducer of apoptosis through activation of caspase-3 [223], we failed to observe activation of the apoptotic marker annexin V and cleavage of caspase-3 in GL cells. Wang et al. studied apoptosis in granulosa cells from rat by Western blot (caspase-3) and nuclear morphology (Hoechst staining) and found no significant effect of treatment by staurosporine [224]. The lack of effect by staurosporine on apoptosis was suggested to be due to differentiation of granulosa cells. Others have found evidence of apoptosis, including cell detachment, membrane shrinkage and formation of apoptotic bodies in luteinized granulosa cells treated with staurosporine [225]. However, Western blot analysis has also revealed activation of several parts of the apoptosis pathway (cleavage of caspase-9, caspase-3, poly-(ADP-ribose)-polymerase (PARP)), in staurosporine-treated cells, suggesting that it may serve as a useful model to delineate the mechanism of apoptosis in the ovary, including the regression of the corpus luteum [225].

In Paper II, we also exposed granulosa-lutein cells to the NF-κB activator phorbol 12-myristate 13-acetate (PMA). PMA is a diester of phorbol and a potent tumor promoter, often employed in biomedical research to activate the signal transduction enzyme protein kinase C PKC [226]. In our study, PMA failed to activate apoptosis in cultured GL cells. One interesting experiment demonstrated that PMA, at several concentrations, stimulated cumulus expansion [227]. Surprisingly, these authors reported that PMA, at low levels, enhanced oocyte competence as reflected by assessments of blastocysts yield (more than 45% blastocysts) [227]. Others detected that PMA at a low concentration mainly act on the cumulus cells, whereas PMA at high concentrations also act on the oocytes [228].

The results of Paper II suggest that AGE and RAGE have a role in ovarian ageing, even though the physical ligands of RAGE and the effect of the up-regulation of RAGEs is still unknown, and the significance of AGE and RAGE in follicular health needs further investigation. Future

research focusing on understanding the clinical effect of AGEs on germ cells, aged oocytes, mitochondrial dysfunction and follicular microenvironment during different stages of development is needed.

5.2.3 Interpretation of results - embryo fragmentation

In Paper III, we aimed to study the determinants of fragmentation during embryo development by assessing the timing of meiotic spindle emergence and the timing of mitotic cell divisions. Although the mechanisms that link delayed meiosis to embryo fragmentation remain speculative, our results indicate that fragmentation may be related to the progress of the meiotic cell cycle and the early mitotic cell cycles. A newly published study reported that human oocytes assembled a meiotic spindle independently of either centrosomes or other microtubule organizing centers (MTOCs), which render efficient spindle assembly and chromosome segregation [229]. The authors reported a surprisingly slow (~ 16 hours) and gradual build-up of a bipolar spindle with aligned chromosomes in human oocytes in contrast to mitosis or oocytes from mice [229]. The spindle assembly period was characterized by spindle instability and abnormal kinetochore-microtubule attachments, which may cause chromosome segregation errors and may explain the high rates of aneuploidy in human oocytes [229]. The timing of the process of fragmentation in relation to the cell cycle has also been studied by Alikani and collaborators. Their results show that fragmentation does not occur in mitotically inactive cells, but emerges in actively dividing cells during cytokinesis, probably in response to altered cytoskeletal organization [130]. Others has concluded that fragmentation of oocytes is a result of an apoptotic process from DNA damage, such as failure of chromosomes and/or centromeres to form a functional meiotic spindle [230].

It is well known that highly fragmented embryos are often chromosomally abnormal [133], raising the possibility that fragmentation was associated with ageing-related aneuploidies. Therefore we also examined, using data in Paper III, whether the spindle emergence and time-lapse time-points were correlating with age.

Battaglia et al. has previously reported that oocytes derived form aged women display abnormal spindles [231]. However, we found no correlation between the women's age and the

appearance of the meiotic spindle by polarized light microscopy (Figure 6). Time-lapse recordings also failed to uncover correlation between advanced age and morphokinetics (Figure 7 and Figure 8).

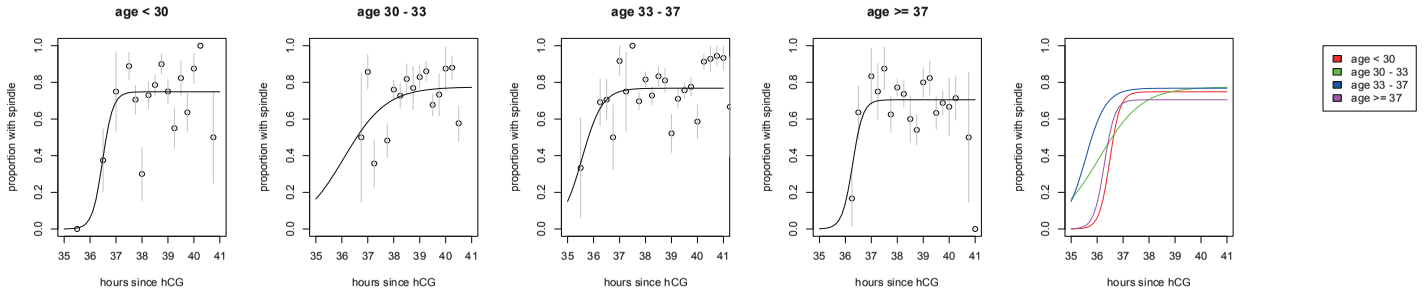

Figure 6: Visualization of the meiotic spindles assessed by polarized light microscopy failed to connect female age to the appearance of the spindles after hCG administration

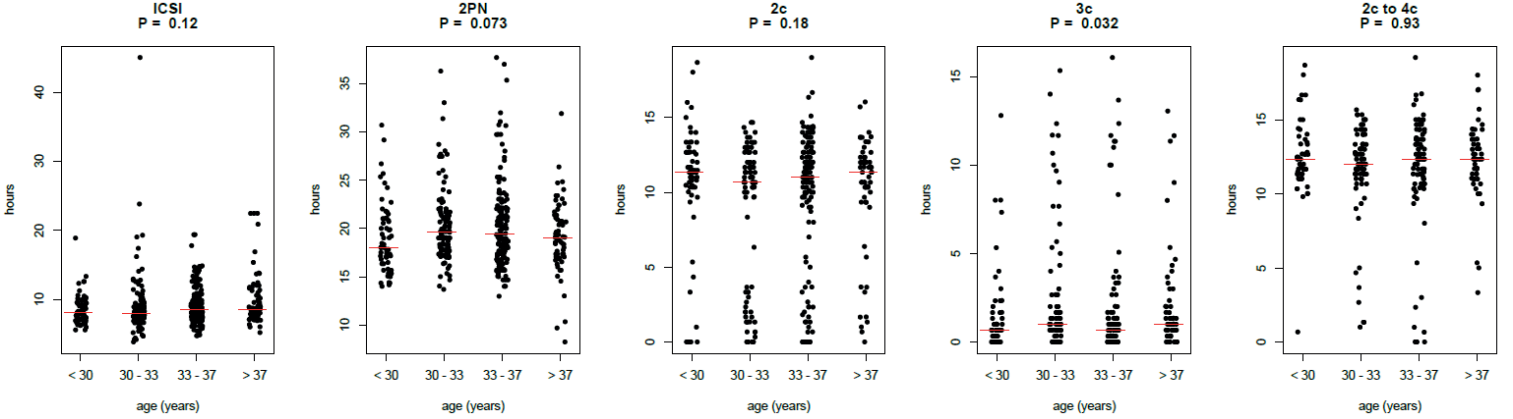

Figure 7: Time-lapse parameters fail to detect age-related influence on embryo development.

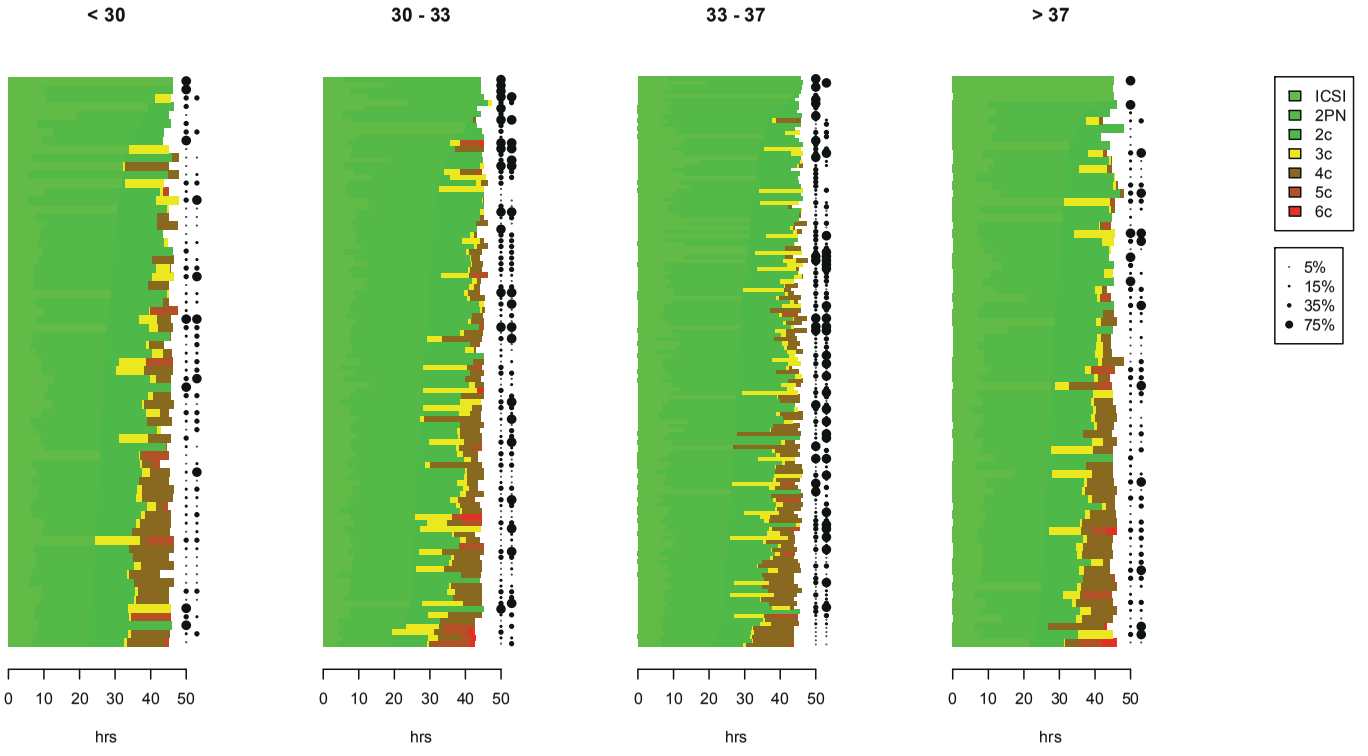

Figure 8: Summary of morphokinetic parameters, included fragmentation, related to female age.

These results may indicate that the abnormalities in human embryos connected to fragmentation may involve different processes than the mechanisms causing abnormalities in oocytes and embryos from aged women.

Reduced spindle retardance has been associated with poor embryo quality of aged patients, although, in general, no association was apparent between retardance and embryo development [232]. Reduced percentage of visible spindle and reduced mean spindle retardance were shown to be related to age [233]. Oocytes derived from aged mice have been found to have a more prolonged meiosis I and increasingly failed to progress to MII compared to oocytes from young mice [234]. There have also been reports of an accelerated meiosis I, increase of abnormal meiosis II, spontaneous oocyte activation, and cellular fragmentation in oocytes derived from aged mice [16, 235].

Oocytes from women of advanced age may have cytoplasmic and nuclear maturation asynchrony due to derangements of hormonal homeostasis, size of the follicle pool, or interaction between oocytes and somatic compartments, which may cause errors in chromosome segregation and effect meiosis and effect fertility [16]. Loss of co-ordination between nuclear maturation and cytokinesis may suggest age-related relaxed cell cycle control [236]. MAD2 is a checkpoint protein known to play a crucial role in meiosis I. Overexpression of MAD2 in meiosis I may lead to cell cycle arrest in metaphase I in mouse oocytes, and downregulation of MAD2 is correlated with a shortened duration of meiosis I and increased oocyte aneuploidy [42, 237]. The impairment of checkpoint function and loss of chromatid cohesion caused by reduced transcription of MAD2 and the cohesin SMCβ1 found in aged oocytes from human and mice, are suggested to contribute to age-related aneuploidy [236, 238]. Additionally, analysis of gene expression in human oocytes found that expression of genes related to cell cycle regulation, cytoskeletal structure, energy pathways, transcription control, and stress responses, are influenced by maternal age [239].

6. Conclusions and future perspectives

Emergence of aneuploidy in oocytes from ageing women appears to be the result of several events rather than an insufficiency in a single mechanism. Reduced number and distribution of chiasmata formed during early prophase I together with weakened centromeric cohesion are probable causes of ovarian ageing. This, in combination with the absence or insufficiency of mechanisms to detect and correct erroneously attached chromosomes in oocytes, results in an increased rate of aneuploidy. Our results suggest that the abnormalities involved in fragmentation of human embryos are distinct from abnormalities seen in oocytes and embryos from aged women.

Our findings on the presence of advanced glycation end products on follicular-derived cells suggest that AGEs may be involved in decline of ovarian function. We propose that AGEs in form of structural long-lived extracellular matrix (ECM) proteins, rather than soluble AGEs may play a role in ovarian ageing, which suggests promising candidates for future research. In particular, modification of long-lived structural proteins in the ECM and on the cell surface, their putative role of activation of inflammation, and the protective role of sRAGE should be examined.

At present, there is no consensus on the optimal way to determine the competence of human embryos derived from in vitro fertilization or to select the most competent embryos for transfer despite recent advances in both invasive and non-invasive techniques. Our results imply that non-invasive technologies used in assisted reproduction laboratories, including morphology assessments, morphokinetics, and meiotic spindle evaluations with polarized light, fail to detect age-related impairment of oocytes and embryos.

As women continue to postpone the birth of their first child, the society should dedicate more attention to the effects of ageing on fertility, which may mandate ovarian reserve assessment, patient education, and early intervention.

7. References

1. Statistics Norway (Statistisk sentralbyrå, SSB): http://www.ssb.no/

2. Broekmans,F.J., Knauff,E.A., te Velde,E.R., Macklon,N.S., & Fauser,B.C. Female reproductive ageing: current knowledge and future trends. Trends Endocrinol. Metab 18, 58-65 (2007).

3. Eijkemans,M.J. et al. Too old to have children? Lessons from natural fertility populations. Hum. Reprod. 29, 1304-1312 (2014).

4. te Velde,E.R. & Pearson,P.L. The variability of female reproductive ageing. Hum. Reprod. Update. 8, 141-154 (2002).

5. Nikolaou,D. & Templeton,A. Early ovarian ageing. Eur. J. Obstet. Gynecol. Reprod. Biol. 113, 126-133 (2004).

6. Hassold,T. & Hunt,P. To err (meiotically) is human: the genesis of human aneuploidy. Nat. Rev. Genet. 2, 280-291 (2001).

7. Hassold,T., Hall,H., & Hunt,P. The origin of human aneuploidy: where we have been, where we are going. Hum. Mol. Genet. 16 Spec No. 2, R203-R208 (2007).

8. Nagaoka,S.I., Hassold,T.J., & Hunt,P.A. Human aneuploidy: mechanisms and new insights into an age-old problem. Nat. Rev. Genet. 13, 493-504 (2012).

9. Fragouli,E., Wells,D., & Delhanty,J.D. Chromosome abnormalities in the human oocyte. Cytogenet. Genome Res. 133, 107-118 (2011).

10. Fragouli,E. & Wells,D. Aneuploidy in the human blastocyst. Cytogenet. Genome Res. 133, 149-159 (2011).

11. Zuckerman, S. The number of oocytes in the mature ovary. Recent Prog Horm Res. 6, 63-109 (1951).

12. Johnson,J., Canning,J., Kaneko,T., Pru,J.K., & Tilly,J.L. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428, 145-150 (2004).

13. White,Y.A. et al. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat. Med. 18, 413-421 (2012).

14. Fragouli,E. et al. The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy. Mol. Hum. Reprod. 17, 286-295 (2011).

15. Henderson,S.A. & Edwards,R.G. Chiasma frequency and maternal age in mammals. Nature 218, 22-28 (1968).

16. Eichenlaub-Ritter,U. Genetics of oocyte ageing. Maturitas 30, 143-169 (1998).

17. Zheng,C.J. & Byers,B. Oocyte selection: a new model for the maternal-age dependence of Down syndrome. Hum. Genet. 90, 1-6 (1992).

18. Warburton,D. Biological aging and the etiology of aneuploidy. Cytogenet. Genome Res. 111, 266-272 (2005).

19. Lamb,N.E. et al. Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II. Nat. Genet. 14, 400-405 (1996).

20. Warren,W.D. & Gorringe,K.L. A molecular model for sporadic human aneuploidy. Trends Genet. 22, 218-224 (2006).

21. Eichenlaub-Ritter,U., Vogt,E., Yin,H., & Gosden,R. Spindles, mitochondria and redox potential in ageing oocytes. Reprod. Biomed. Online. 8, 45-58 (2004).

22. Kleckner,N. Chiasma formation: chromatin/axis interplay and the role(s) of the synaptonemal complex. Chromosoma 115, 175-194 (2006).

23. Walker,M.Y. & Hawley,R.S. Hanging on to your homolog: the roles of pairing, synapsis and recombination in the maintenance of homolog adhesion. Chromosoma 109, 3-9 (2000).

24. Ozturk,S., Sozen,B., & Demir,N. Telomere length and telomerase activity during oocyte maturation and early embryo development in mammalian species. Mol. Hum. Reprod. 20, 15-30 (2014).

25. Page,S.L. & Hawley,R.S. Chromosome choreography: the meiotic ballet. Science 301, 785-789 (2003).

26. Lamb,N.E. et al. Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II. Nat. Genet. 14, 400-405 (1996).

27. Jones,K.T. Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. Hum. Reprod. Update. 14, 143-158 (2008).

28. Nasmyth,K. & Haering,C.H. Cohesin: its roles and mechanisms. Annu. Rev. Genet. 43, 525-558 (2009).

29. Buonomo,S.B. et al. Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. Cell 103, 387-398 (2000).

30. Tachibana-Konwalski,K. et al. Rec8-containing cohesin maintains bivalents without turnover during the growing phase of mouse oocytes. Genes Dev. 24, 2505-2516 (2010).

31. Lister,L.M. et al. Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. Curr. Biol. 20, 1511-1521 (2010).

32. Hodges,C.A., Revenkova,E., Jessberger,R., Hassold,T.J., & Hunt,P.A. SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction. Nat. Genet. 37, 1351-1355 (2005).

33. Kitajima,T.S. et al. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature 441, 46-52 (2006).

34. Tanno,Y. et al. Phosphorylation of mammalian Sgo2 by Aurora B recruits PP2A and MCAK to centromeres. Genes Dev. 24, 2169-2179 (2010).

35. Ishiguro,T., Tanaka,K., Sakuno,T., & Watanabe,Y. Shugoshin-PP2A counteracts casein-kinase-1-dependent cleavage of Rec8 by separase. Nat. Cell Biol. 12, 500-506 (2010).

36. Xu,Z. et al. Structure and function of the PP2A-shugoshin interaction. Mol. Cell 35, 426-441 (2009).

37. Shao,H. et al. Aurora B regulates spindle bipolarity in meiosis in vertebrate oocytes. Cell Cycle 11, 2672-2680 (2012).

38. Angell,R.R. Predivision in human oocytes at meiosis I: a mechanism for trisomy formation in man. Hum. Genet. 86, 383-387 (1991).

39. Hauf,S. & Watanabe,Y. Kinetochore orientation in mitosis and meiosis. Cell 119, 317-327 (2004).

40. Handyside,A.H. et al. Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation. Eur. J. Hum. Genet. 20, 742-747 (2012).

41. Musacchio,A. & Hardwick,K.G. The spindle checkpoint: structural insights into dynamic signalling. Nat. Rev. Mol. Cell Biol. 3, 731-741 (2002).

42. Homer,H.A. Mad2 and spindle assembly checkpoint function during meiosis I in mammalian oocytes. Histol. Histopathol. 21, 873-886 (2006).

43. Musacchio,A. & Salmon,E.D. The spindle-assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. 8, 379-393 (2007).

44. Musacchio,A. Spindle assembly checkpoint: the third decade. Philos. Trans. R. Soc. Lond B Biol. Sci. 366, 3595-3604 (2011).

45. DeLuca,J.G. & Musacchio,A. Structural organization of the kinetochore-microtubule interface. Curr. Opin. Cell Biol. 24, 48-56 (2012).

46. Nicklas,R.B. & Arana,P. Evolution and the meaning of metaphase. J. Cell Sci. 102, 681-690 (1992).

47. Malmanche,N., Maia,A., & Sunkel,C.E. The spindle assembly checkpoint: preventing chromosome mis-segregation during mitosis and meiosis. FEBS Lett. 580, 2888-2895 (2006).

48. Lampson,M.A. & Cheeseman,I.M. Sensing centromere tension: Aurora B and the regulation of kinetochore function. Trends Cell Biol. 21, 133-140 (2011).

49. Kouznetsova,A., Lister,L., Nordenskjold,M., Herbert,M., & Hoog,C. Bi-orientation of achiasmatic chromosomes in meiosis I oocytes contributes to aneuploidy in mice. Nat. Genet. 39, 966-968 (2007).

50. Sebestova,J., Danylevska,A., Novakova,L., Kubelka,M., & Anger,M. Lack of response to unaligned chromosomes in mammalian female gametes. Cell Cycle 11, 3011-3018 (2012).

51. Vogt,E., Kirsch-Volders,M., Parry,J., & Eichenlaub-Ritter,U. Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mutat. Res. 651, 14-29 (2008).

52. Schultz,R.M. From egg to embryo: a peripatetic journey. Reproduction. 130, 825-828 (2005).

53. Hamatani,T. et al. Age-associated alteration of gene expression patterns in mouse oocytes. Hum. Mol. Genet. 13, 2263-2278 (2004).

54. Wright,D.L. et al. Characterization of telomerase activity in the human oocyte and preimplantation embryo. Mol. Hum. Reprod. 7, 947-955 (2001).

55. Liu,L. et al. Telomere lengthening early in development. Nat. Cell Biol. 9, 1436-1441 (2007).

56. Keefe,D.L., Liu,L., & Marquard,K. Telomeres and aging-related meiotic dysfunction in women. Cell Mol. Life Sci. 64, 139-143 (2007).

57. Kalmbach,K.H. et al. Telomeres and human reproduction. Fertil. Steril. 99, 23-29 (2013).

58. Tatone,C. et al. Cellular and molecular aspects of ovarian follicle ageing. Hum. Reprod. Update. 14, 131-142 (2008).

59. Thorpe,S.R. & Baynes,J.W. Role of the Maillard reaction in diabetes mellitus and diseases of aging. Drugs Aging 9, 69-77 (1962)

60. Maillard, L.C. Action des acides amines sur les sucres: formation des melanoidines par voie methodique. C. R. Acad. Sci. 154, 66− 68 (1912).

61. Brownlee,M., Vlassara,H., & Cerami,A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann. Intern. Med. 101, 527-537 (1984).

62. Brownlee,M., Vlassara,H., & Cerami,A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann. Intern. Med. 101, 527-537 (1984).

63. Kirstein,M. et al. Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. Proc. Natl. Acad. Sci. U. S. A 87, 9010-9014 (1990).

64. Vitek,M.P. et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A 91, 4766-4770 (1994).

65. Luevano-Contreras,C. & Chapman-Novakofski,K. Dietary advanced glycation end products and aging. Nutrients. 2, 1247-1265 (2010).

66. Brownlee,M. Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813-820 (2001).

67. Piperi,C., Adamopoulos,C., Dalagiorgou,G., Diamanti-Kandarakis,E., & Papavassiliou,A.G. Crosstalk between advanced glycation and endoplasmic reticulum stress: emerging therapeutic targeting for metabolic diseases. J. Clin. Endocrinol. Metab 97, 2231-2242 (2012).

68. Kalea,A.Z., Schmidt,A.M., & Hudson,B.I. RAGE: a novel biological and genetic marker for vascular disease. Clin. Sci. (Lond) 116, 621-637 (2009).

69. Basta,G. Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications. Atherosclerosis 196, 9-21 (2008).

70. Yan,S.F., D'Agati,V., Schmidt,A.M., & Ramasamy,R. Receptor for Advanced Glycation Endproducts (RAGE): a formidable force in the pathogenesis of the cardiovascular complications of diabetes & aging. Curr. Mol. Med. 7, 699-710 (2007).

71. Merhi,Z. Advanced glycation end products and their relevance in female reproduction. Hum. Reprod. 29, 135-145 (2014).

72. Goldberg,T. et al. Advanced glycoxidation end products in commonly consumed foods. J. Am. Diet. Assoc. 104, 1287-1291 (2004).

73. Vlassara,H. & Palace,M.R. Glycoxidation: the menace of diabetes and aging. Mt. Sinai J. Med. 70, 232-241 (2003).

74. Diamanti-Kandarakis,E. et al. Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries. Histochem. Cell Biol. 127, 581-589 (2007).

75. Fujii,E.Y., Nakayama,M., & Nakagawa,A. Concentrations of receptor for advanced glycation end products, VEGF and CML in plasma, follicular fluid, and peritoneal fluid in women with and without endometriosis. Reprod. Sci. 15, 1066-1074 (2008).

76. Tatone,C. & Amicarelli,F. The aging ovary--the poor granulosa cells. Fertil. Steril. 99, 12-17 (2013).

77. Vlassara,H. & Palace,M.R. Diabetes and advanced glycation endproducts. J. Intern. Med. 251, 87-101 (2002).

78. Diamanti-Kandarakis,E., Piperi,C., Kalofoutis,A., & Creatsas,G. Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome. Clin. Endocrinol. (Oxf) 62, 37-43 (2005).

79. Uribarri,J. et al. Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. J. Gerontol. A Biol. Sci. Med. Sci. 62, 427-433 (2007).

80. Bruckova,L. et al. The cultivation of human granulosa cells. Acta Medica. (Hradec. Kralove) 51, 165-172 (2008).

81. Perry,G.A. et al. Relationship between follicle size at insemination and pregnancy success. Proc. Natl. Acad. Sci. U. S. A 102, 5268-5273 (2005).

82. Jozwik,M. & Wolczynski,S. Technical note: blood contamination of aspirated follicular fluid. J. Assist. Reprod. Genet. 15, 409-410 (1998).

83. Levay,P.F., Huyser,C., Fourie,F.L., & Rossouw,D.J. The detection of blood contamination in human follicular fluid. J. Assist. Reprod. Genet. 14, 212-217 (1997).

84. Beckmann,M.W., Polacek,D., Seung,L., & Schreiber,J.R. Human ovarian granulosa cell culture: determination of blood cell contamination and evaluation of possible culture purification steps. Fertil. Steril. 56, 881-887 (1991).

85. Fedorcsak,P., Raki,M., & Storeng,R. Characterization and depletion of leukocytes from cells isolated from the pre-ovulatory ovarian follicle. Hum. Reprod. 22, 989-994 (2007).

86. Figenschau,Y. et al. A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells. Hum. Reprod. 12, 523-531 (1997).

87. Smith,M.P., Flannery,G.R., Randle,B.J., Jenkins,J.M., & Holmes,C.H. Leukocyte origin and profile in follicular aspirates at oocyte retrieval. Hum. Reprod. 20, 3526-3531 (2005).

88. Lachapelle,M.H., Hemmings,R., Roy,D.C., Falcone,T., & Miron,P. Flow cytometric evaluation of leukocyte subpopulations in the follicular fluids of infertile patients. Fertil. Steril. 65, 1135-1140 (1996).

89. Fedorcsak,P. et al. Differential release of matrix metalloproteinases and tissue inhibitors of metalloproteinases by human granulosa-lutein cells and ovarian leukocytes. Endocrinology 151, 1290-1298 (2010).

90. Polec,A., Tanbo,T., & Fedorcsak,P. Cellular interaction regulates interleukin-8 secretion by granulosa-lutein cells and monocytes/macrophages. Am. J. Reprod. Immunol. 61, 85-94 (2009).

91. Seifer,D.B., DeJesus,V., & Hubbard,K. Mitochondrial deletions in luteinized granulosa cells as a function of age in women undergoing in vitro fertilization. Fertil. Steril. 78, 1046-1048 (2002).

92. Tatone,C. et al. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. Mol. Hum. Reprod. 12, 655-660 (2006).

93. Pellicer,A. et al. Effects of aging on the human ovary: the secretion of immunoreactive alpha-inhibin and progesterone. Fertil. Steril. 61, 663-668 (1994).

94. Seifer,D.B. et al. Autologous granulosa cell coculture demonstrates zygote suppression of granulosa cell steroidogenesis. Fertil. Steril. 66, 425-429 (1996).

95. Leridon,H. Can assisted reproduction technology compensate for the natural decline in fertility with age? A model assessment. Hum. Reprod. 19, 1548-1553 (2004).

96. Sunkara,S.K. et al. Association between the number of eggs and live birth in IVF treatment: an analysis of 400 135 treatment cycles. Hum. Reprod. 26, 1768-1774 (2011).

97. Lashen,H. & Ledger,W. Management of poor responders in IVF. Hum. Reprod. 14, 1919 (1999).

98. Bancsi,L.F. et al. Basal follicle-stimulating hormone levels are of limited value in predicting ongoing pregnancy rates after in vitro fertilization. Fertil. Steril. 73, 552-557 (2000).

99. Ebbesen,S.M. et al. Stressful life events are associated with a poor in-vitro fertilization (IVF) outcome: a prospective study. Hum. Reprod. 24, 2173-2182 (2009).

100. Hauzman,E. et al. Use of serum inhibin A and human chorionic gonadotropin measurements to predict the outcome of in vitro fertilization pregnancies. Fertil. Steril. 81, 66-72 (2004).

101. Ferlitsch,K. et al. Body mass index, follicle-stimulating hormone and their predictive value in in vitro fertilization. J. Assist. Reprod. Genet. 21, 431-436 (2004).

102. Hart,R. et al. A prospective controlled study of the effect of intramural uterine fibroids on the outcome of assisted conception. Hum. Reprod. 16, 2411-2417 (2001).

103. Ferraretti,A.P. et al. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum. Reprod. 26, 1616-1624 (2011).

104. Braude,P. Selecting the 'best' embryos: prospects for improvement. Reprod. Biomed. Online. 27, 644-653 (2013).

105. Killackey,M.A. & Neuwirth,R.S. Evaluation and management of the pelvic mass: a review of 540 cases. Obstet. Gynecol. 71, 319-322 (1988).

106. Cleary-Goldman,J. et al. Impact of maternal age on obstetric outcome. Obstet. Gynecol. 105, 983-990 (2005).

107. Barlow,P. et al. In vitro fertilization, development, and implantation after exposure of mature mouse oocytes to visible light. Mol. Reprod. Dev. 33, 297-302 (1992).

108. Bedford,J.M. & Dobrenis,A. Light exposure of oocytes and pregnancy rates after their transfer in the rabbit. J. Reprod. Fertil. 85, 477-481 (1989).

109. Payne,D., Flaherty,S.P., Barry,M.F., & Matthews,C.D. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Hum. Reprod. 12, 532-541 (1997).

110. Zigler,J.S., Jr., Lepe-Zuniga,J.L., Vistica,B., & Gery,I. Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. In Vitro Cell Dev. Biol. 21, 282-287 (1985).

111. Cummins,J.M. et al. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. J. In Vitro Fert. Embryo. Transf. 3, 284-295 (1986).

112. Gardner,D.K. & Schoolcraft,W.B. Culture and transfer of human blastocysts. Curr. Opin. Obstet. Gynecol. 11, 307-311 (1999).

113. Puissant,F., Van,R.M., Barlow,P., Deweze,J., & Leroy,F. Embryo scoring as a prognostic tool in IVF treatment. Hum. Reprod. 2, 705-708 (1987).

114. Van,R.E. et al. Characterization of a top quality embryo, a step towards single-embryo transfer. Hum. Reprod. 14, 2345-2349 (1999).

115. Van,R.E. et al. Multinucleation in cleavage stage embryos. Hum. Reprod. 18, 1062-1069 (2003).

116. Ziebe,S. et al. FISH analysis for chromosomes 13, 16, 18, 21, 22, X and Y in all blastomeres of IVF pre-embryos from 144 randomly selected donated human oocytes and impact on pre-embryo morphology. Hum. Reprod. 18, 2575-2581 (2003).

117. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum. Reprod. 26, 1270-1283 (2011).

118. Ziebe,S. et al. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum. Reprod. 12, 1545-1549 (1997).

119. Rhenman,A. et al. Which set of embryo variables is most predictive for live birth? A prospective study in 6252 single embryo transfers to construct an embryo score for the ranking and selection of embryos. Hum. Reprod. 30, 28-36 (2015).

120. Edwards,R.G. et al. Factors influencing the success of in vitro fertilization for alleviating human infertility. J. In Vitro Fert. Embryo. Transf. 1, 3-23 (1984).

121. Hesters,L. et al. Impact of early cleaved zygote morphology on embryo development and in vitro fertilization-embryo transfer outcome: a prospective study. Fertil. Steril. 89, 1677-1684 (2008).

122. Lundin,K., Bergh,C., & Hardarson,T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum. Reprod. 16, 2652-2657 (2001).

123. Tesarik,J., Kopecny,V., Plachot,M., & Mandelbaum,J. Ultrastructural and autoradiographic observations on multinucleated blastomeres of human cleaving embryos obtained by in-vitro fertilization. Hum. Reprod. 2, 127-136 (1987).

124. Staessen,C. & Van,S.A. The genetic constitution of multinuclear blastomeres and their derivative daughter blastomeres. Hum. Reprod. 13, 1625-1631 (1998).

125. Hardarson,T., Hanson,C., Sjogren,A., & Lundin,K. Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. Hum. Reprod. 16, 313-318 (2001).

126. Scott,L., Finn,A., O'Leary,T., McLellan,S., & Hill,J. Morphologic parameters of early cleavage-stage embryos that correlate with fetal development and delivery: prospective and applied data for increased pregnancy rates. Hum. Reprod. 22, 230-240 (2007).

127. Johansson,M., Hardarson,T., & Lundin,K. There is a cutoff limit in diameter between a blastomere and a small anucleate fragment. J. Assist. Reprod. Genet. 20, 309-313 (2003).

128. Hardarson,T. et al. Internalization of cellular fragments in a human embryo: time-lapse recordings. Reprod. Biomed. Online. 5, 36-38 (2002).

129. Alikani,M. et al. Human embryo fragmentation in vitro and its implications for pregnancy and implantation. Fertil. Steril. 71, 836-842 (1999).

130. Alikani,M., Schimmel,T., & Willadsen,S.M. Cytoplasmic fragmentation in activated eggs occurs in the cytokinetic phase of the cell cycle, in lieu of normal cytokinesis, and in response to cytoskeletal disorder. Mol. Hum. Reprod. 11, 335-344 (2005).

131. Wong,C.C. et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat. Biotechnol. 28, 1115-1121 (2010).

132. Magli,M.C. et al. Embryo morphology and development are dependent on the chromosomal complement. Fertil. Steril. 87, 534-541 (2007).

133. Munne,S. Chromosome abnormalities and their relationship to morphology and development of human embryos. Reprod. Biomed. Online. 12, 234-253 (2006).

134. Liu,J. et al. DNA microarray reveals that high proportions of human blastocysts from women of advanced maternal age are aneuploid and mosaic. Biol. Reprod. 87, 148 (2012).

135. Montag,M., Koster,M., van,d., V, & van,d., V Gamete competence assessment by polarizing optics in assisted reproduction. Hum. Reprod. Update. 17, 654-666 (2011).

136. Oldenbourg,R. & Mei,G. New polarized light microscope with precision universal compensator. J. Microsc. 180, 140-147 (1995).

137. Keefe,D., Tran,P., Pellegrini,C., & Oldenbourg,R. Polarized light microscopy and digital image processing identify a multilaminar structure of the hamster zona pellucida. Hum. Reprod. 12, 1250-1252 (1997).

138. Silva,C.P., Kommineni,K., Oldenbourg,R., & Keefe,D.L. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. Fertil. Steril. 71, 719-721 (1999).

139. Keefe,D., Liu,L., Wang,W., & Silva,C. Imaging meiotic spindles by polarization light microscopy: principles and applications to IVF. Reprod. Biomed. Online. 7, 24-29 (2003).

140. Wang,W.H., Meng,L., Hackett,R.J., Odenbourg,R., & Keefe,D.L. The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. Fertil. Steril. 75, 348-353 (2001).

141. Montag,M., Schimming,T., & van,d., V Spindle imaging in human oocytes: the impact of the meiotic cell cycle. Reprod. Biomed. Online. 12, 442-446 (2006).

142. Moon,J.H. et al. Spindle positions and their distributions in in vivo and in vitro matured mouse oocytes. Hum. Reprod. 20, 2207-2210 (2005).

143. Wang,W.H. & Keefe,D.L. Prediction of chromosome misalignment among in vitro matured human oocytes by spindle imaging with the PolScope. Fertil. Steril. 78, 1077-1081 (2002).

144. Wang,W.H., Meng,L., Hackett,R.J., Oldenbourg,R., & Keefe,D.L. Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. Fertil. Steril. 77, 1274-1277 (2002).

145. Cohen,Y. et al. Spindle imaging: a new marker for optimal timing of ICSI? Hum. Reprod. 19, 649-654 (2004).

146. Shen,Y., Betzendahl,I., Tinneberg,H.R., & Eichenlaub-Ritter,U. Enhanced polarizing microscopy as a new tool in aneuploidy research in oocytes. Mutat. Res. 651, 131-140 (2008).

147. Eichenlaub-Ritter,U., Shen,Y., & Tinneberg,H.R. Manipulation of the oocyte: possible damage to the spindle apparatus. Reprod. Biomed. Online. 5, 117-124 (2002).

148. Beall,S., Brenner,C., & Segars,J. Oocyte maturation failure: a syndrome of bad eggs. Fertil. Steril. 94, 2507-2513 (2010).

149. Madgwick,S. & Jones,K.T. How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor. Cell Div. 2, 4 (2007).

150. Madgwick,S., Hansen,D.V., Levasseur,M., Jackson,P.K., & Jones,K.T. Mouse Emi2 is required to enter meiosis II by reestablishing cyclin B1 during interkinesis. J. Cell Biol. 174, 791-801 (2006).

151. Kirkegaard,K., Hindkjaer,J.J., Grondahl,M.L., Kesmodel,U.S., & Ingerslev,H.J. A randomized clinical trial comparing embryo culture in a conventional incubator with a time-lapse incubator. J. Assist. Reprod. Genet. 29, 565-572 (2012).

152. Kirkegaard,K., Agerholm,I.E., & Ingerslev,H.J. Time-lapse monitoring as a tool for clinical embryo assessment. Hum. Reprod. 27, 1277-1285 (2012).

153. Meseguer,M. et al. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. Fertil. Steril. 98, 1481-1489 (2012).

154. Wong,C., Chen,A.A., Behr,B., & Shen,S. Time-lapse microscopy and image analysis in basic and clinical embryo development research. Reprod. Biomed. Online. 26, 120-129 (2013).

155. Armstrong,S., Vail,A., Mastenbroek,S., Jordan,V., & Farquhar,C. Time-lapse in the IVF-lab: how should we assess potential benefit? Hum. Reprod. 30, 3-8 (2015).

156. Armstrong,S., Arroll,N., Cree,L.M., Jordan,V., & Farquhar,C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. Cochrane. Database. Syst. Rev. 2, CD011320 (2015).

157. Rienzi,L. et al. No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: a longitudinal cohort study. Reprod. Biomed. Online. 30, 57-66 (2015).

158. Ottolini,C., Rienzi,L., & Capalbo,A. A cautionary note against embryo aneuploidy risk assessment using time-lapse imaging. Reprod. Biomed. Online. 28, 273-275 (2014).

159. Basile,N. et al. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. Fertil. Steril. 101, 699-704 (2014).

160. Swain,J.E. Could time-lapse embryo imaging reduce the need for biopsy and PGS? J. Assist. Reprod. Genet. 30, 1081-1090 (2013).

161. Montag,M., Toth,B., & Strowitzki,T. New approaches to embryo selection. Reprod. Biomed. Online. 27, 539-546 (2013).

162. Paternot,G., Devroe,J., Debrock,S., D'Hooghe,T.M., & Spiessens,C. Intra- and inter-observer analysis in the morphological assessment of early-stage embryos. Reprod. Biol. Endocrinol. 7, 105 (2009).

163. Guerif,F. et al. Limited value of morphological assessment at days 1 and 2 to predict blastocyst development potential: a prospective study based on 4042 embryos. Hum. Reprod. 22, 1973-1981 (2007).

164. Fragouli,E., Alfarawati,S., Spath,K., & Wells,D. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. Mol. Hum. Reprod. 20, 117-126 (2014).

165. Roux,C. et al. Morphometric parameters of living human in-vitro fertilization embryos; importance of the asynchronous division process. Hum. Reprod. 10, 1201-1207 (1995).

166. Arce,J.C. et al. Interobserver agreement and intraobserver reproducibility of embryo quality assessments. Hum. Reprod. 21, 2141-2148 (2006).

167. Baxter Bendus,A.E., Mayer,J.F., Shipley,S.K., & Catherino,W.H. Interobserver and intraobserver variation in day 3 embryo grading. Fertil. Steril. 86, 1608-1615 (2006).

168. Garrisi,G.J. et al. Analysis of factors contributing to success in a program of micromanipulation-assisted fertilization. Fertil. Steril. 59, 366-374 (1993).

169. Akarsu,C., Caglar,G., Vicdan,K., Sozen,E., & Biberoglu,K. Smooth endoplasmic reticulum aggregations in all retrieved oocytes causing recurrent multiple anomalies: case report. Fertil. Steril. 92, 1496-3 (2009).

170. Ashourzadeh,S. et al. Noninvasive assays of in vitro matured human oocytes showed insignificant correlation with fertilization and embryo development. Arch. Gynecol. Obstet.(2015).

171. Balaban,B. & Urman,B. Effect of oocyte morphology on embryo development and implantation. Reprod. Biomed. Online. 12, 608-615 (2006).

172. Check,J.H. et al. Oocytes from women of advanced reproductive age do not appear to have an increased risk of zona pellucida hardening. Clin. Exp. Obstet. Gynecol. 39, 440-441 (2012).

173. Ciotti,P.M. et al. First polar body morphology before ICSI is not related to embryo quality or pregnancy rate. Hum. Reprod. 19, 2334-2339 (2004).

174. Ebner,T., Moser,M., & Tews,G. Is oocyte morphology prognostic of embryo developmental potential after ICSI? Reprod. Biomed. Online. 12, 507-512 (2006).

175. Mahadevan,M.M. & Fleetham,J. Relationship of a human oocyte scoring system to oocyte maturity and fertilizing capacity. Int. J. Fertil. 35, 240-244 (1990).

176. Mandelbaum,J. Oocytes. Hum. Reprod. 15 Suppl 4, 11-18 (2000).

177. Hammitt,D.G. et al. Maturational asynchrony between oocyte cumulus-coronal morphology and nuclear maturity in gonadotropin-releasing hormone agonist stimulations. Fertil. Steril. 59, 375-381 (1993).

178. Hammitt,D.G. et al. Prediction of nuclear maturity from cumulus-coronal morphology: influence of embryologist experience. J. Assist. Reprod. Genet. 9, 439-446 (1992).

179. Claman,P. et al. The impact of embryo quality and quantity on implantation and the establishment of viable pregnancies. J. In Vitro Fert. Embryo. Transf. 4, 218-222 (1987).

180. Holte,J. et al. Construction of an evidence-based integrated morphology cleavage embryo score for implantation potential of embryos scored and transferred on day 2 after oocyte retrieval. Hum. Reprod. 22, 548-557 (2007).

181. Munne,S. et al. Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos. Reprod. Biomed. Online. 14, 628-634 (2007).

182. Antczak,M. & Van,B.J. Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. Hum. Reprod. 14, 429-447 (1999).

183. Rienzi,L. et al. Significance of morphological attributes of the early embryo. Reprod. Biomed. Online. 10, 669-681 (2005).

184. Giorgetti,C. et al. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. Hum. Reprod. 10, 2427-2431 (1995).

185. Portela,V.M., Zamberlam,G., & Price,C.A. Cell plating density alters the ratio of estrogenic to progestagenic enzyme gene expression in cultured granulosa cells. Fertil. Steril. 93, 2050-2055 (2010).

186. Andree,H.A. et al. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J. Biol. Chem. 265, 4923-4928 (1990).

187. Lavorato,H.L. et al. GnRH agonist versus GnRH antagonist in IVF/ICSI cycles with recombinant LH supplementation: DNA fragmentation and apoptosis in granulosa cells. Eur. J. Obstet. Gynecol. Reprod. Biol. 165, 61-65 (2012).

188. Jancar,N., Kopitar,A.N., Ihan,A., Virant,K., I, & Bokal,E.V. Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. J. Assist. Reprod. Genet. 24, 91-97 (2007).

189. Tajes,M. et al. Methylglyoxal reduces mitochondrial potential and activates Bax and caspase-3 in neurons: Implications for Alzheimer's disease. Neurosci. Lett. 580, 78-82 (2014).

190. Reber,F., Reber,U., & Funk,R.H. Intracellular changes in astrocytes and NG 108-15 neuroblastoma X glioma cells induced by advanced glycation end products. J. Neural Transm. 110, 1103-1118 (2003).

191. Vaskivuo,T.E. et al. Role of apoptosis, apoptosis-related factors and 17beta-hydroxysteroid dehydrogenases in human corpus luteum regression. Mol. Cell Endocrinol. 194, 191-200 (2002).

192. Onyango,I.G., Tuttle,J.B., & Bennett,J.P., Jr. Altered intracellular signaling and reduced viability of Alzheimer's disease neuronal cybrids is reproduced by beta-amyloid peptide acting through receptor for advanced glycation end products (RAGE). Mol. Cell Neurosci. 29, 333-343 (2005).

193. Rienzi,L., Vajta,G., & Ubaldi,F. Predictive value of oocyte morphology in human IVF: a systematic review of the literature. Hum. Reprod. Update. 17, 34-45 (2011).

194. Coticchio,G. et al. Comparative analysis of the metaphase II spindle of human oocytes through polarized light and high-performance confocal microscopy. Fertil. Steril. 93, 2056-2064 (2010).

195. Stecher,A. et al. Transfer of blastocysts with deviant morphological and morphokinetic parameters at early stages of in-vitro development: a case series. Reprod. Biomed. Online. 28, 424-435 (2014).

196. Kirkegaard,K. et al. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. Reprod. Biomed. Online. 29, 156-158 (2014).

197. Janssen,A., van der Burg,M., Szuhai,K., Kops,G.J., & Medema,R.H. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Science 333, 1895-1898 (2011).

198. Sundvall,L., Ingerslev,H.J., Breth,K.U., & Kirkegaard,K. Inter- and intra-observer variability of time-lapse annotations. Hum. Reprod. 28, 3215-3221 (2013).

199. Ciray,H.N. et al. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Hum. Reprod. 29, 2650-2660 (2014).

200. Li,R. et al. Effect of red light on the development and quality of mammalian embryos. J. Assist. Reprod. Genet. 31, 795-801 (2014).

201. Ziebe,S. et al. Embryo quality and developmental potential is compromised by age. Acta Obstet. Gynecol. Scand. 80, 169-174 (2001).

202. Janny,L. & Menezo,Y.J. Maternal age effect on early human embryonic development and blastocyst formation. Mol. Reprod. Dev. 45, 31-37 (1996).

203. Lim,A.S. & Tsakok,M.F. Age-related decline in fertility: a link to degenerative oocytes? Fertil. Steril. 68, 265-271 (1997).

204. Bar-Hava,I. et al. Does female age affect embryo morphology? Gynecol. Endocrinol. 13, 371-374 (1999).

205. Wu,D.H. et al. Age does not influence the effect of embryo fragmentation on successful blastocyst development. Fertil. Steril. 95, 2778-2780 (2011).

206. Liu,Y. et al. Germline stem cells and neo-oogenesis in the adult human ovary. Dev. Biol. 306, 112-120 (2007).

207. Giorgetti,C. et al. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. Hum. Reprod. 10, 2427-2431 (1995).

208. Eaton,J.L., Hacker,M.R., Barrett,C.B., Thornton,K.L., & Penzias,A.S. Influence of patient age on the association between euploidy and day-3 embryo morphology. Fertil. Steril. 94, 365-367 (2010).

209. Hsu,M.I. et al. Embryo implantation in in vitro fertilization and intracytoplasmic sperm injection: impact of cleavage status, morphology grade, and number of embryos transferred. Fertil. Steril. 72, 679-685 (1999).

210. van Kooij,R.J., Looman,C.W., Habbema,J.D., Dorland,M., & te Velde,E.R. Age-dependent decrease in embryo implantation rate after in vitro fertilization. Fertil. Steril. 66, 769-775 (1996).

211. Papachroni,K.K. et al. Lysyl oxidase interacts with AGE signalling to modulate collagen synthesis in polycystic ovarian tissue. J. Cell Mol. Med. 14, 2460-2469 (2010).

212. Matsumine,M., Shibata,N., Ishitani,K., Kobayashi,M., & Ohta,H. Pentosidine accumulation in human oocytes and their correlation to age-related apoptosis. Acta Histochem. Cytochem. 41, 97-104 (2008).

213. Diamanti-Kandarakis,E. et al. Increased serum advanced glycation end-products is a distinct finding in lean women with polycystic ovary syndrome (PCOS). Clin. Endocrinol. (Oxf) 69, 634-641 (2008).

214. Diamanti-Kandarakis,E., Piperi,C., Kalofoutis,A., & Creatsas,G. Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome. Clin. Endocrinol. (Oxf) 62, 37-43 (2005).

215. Sharma,I., Dhawan,V., Saha,S.C., Rashmi,B., & Dhaliwal,L.K. Implication of the RAGE-EN-RAGE axis in endometriosis. Int. J. Gynaecol. Obstet. 110, 199-202 (2010).

216. Ota,K. et al. Elevation of soluble form of receptor for advanced glycation end products (sRAGE) in recurrent pregnancy losses (RPL): possible participation of RAGE in RPL. Fertil. Steril. 102, 782-789 (2014).

217. Germanova,A. et al. Soluble receptor for advanced glycation end products in physiological and pathological pregnancy. Clin. Biochem. 43, 442-446 (2010).

218. Kwon,J.H., Kim,Y.H., Kwon,J.Y., & Park,Y.W. Clinical significance of serum sRAGE and esRAGE in women with normal pregnancy and preeclampsia. J. Perinat. Med. 39, 507-513 (2011).

219. Merhi,Z., Irani,M., Doswell,A.D., & Ambroggio,J. Follicular fluid soluble receptor for advanced glycation end-products (sRAGE): a potential indicator of ovarian reserve. J. Clin. Endocrinol. Metab 99, E226-E233 (2014).

220. Malickova,K. et al. Concentrations of sRAGE in serum and follicular fluid in assisted reproductive cycles--a preliminary study. Clin. Lab 56, 377-384 (2010).

221. Fujii,E.Y. & Nakayama,M. The measurements of RAGE, VEGF, and AGEs in the plasma and follicular fluid of reproductive women: the influence of aging. Fertil. Steril. 94, 694-700 (2010).

222. Jinno,M. et al. Advanced glycation end-products accumulation compromises embryonic development and achievement of pregnancy by assisted reproductive technology. Hum. Reprod. 26, 604-610 (2011).

223. Chae,H.J. et al. Molecular mechanism of staurosporine-induced apoptosis in osteoblasts. Pharmacol. Res. 42, 373-381 (2000).

224. Wang,Q., Leader,A., & Tsang,B.K. Follicular stage-dependent regulation of apoptosis and steroidogenesis by prohibitin in rat granulosa cells. J. Ovarian. Res. 6, 23 (2013).

225. Khan,S.M., Dauffenbach,L.M., & Yeh,J. Mitochondria and caspases in induced apoptosis in human luteinized granulosa cells. Biochem. Biophys. Res. Commun. 269, 542-545 (2000).

226. Sriraman,V., Modi,S.R., Bodenburg,Y., Denner,L.A., & Urban,R.J. Identification of ERK and JNK as signaling mediators on protein kinase C activation in cultured granulosa cells. Mol. Cell Endocrinol. 294, 52-60 (2008).

227. Ali,A. & Sirard,M.A. Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. Reproduction. 130, 303-310 (2005).

228. Su,Y.Q., Xia,G.L., Byskov,A.G., Fu,G.D., & Yang,C.R. Protein kinase C and intracellular calcium are involved in follicle-stimulating hormone-mediated meiotic resumption of cumulus cell-enclosed porcine oocytes in hypoxanthine-supplemented medium. Mol. Reprod. Dev. 53, 51-58 (1999).

229. Holubcova,Z., Blayney,M., Elder,K., & Schuh,M. Human oocytes. Error-prone chromosome-mediated spindle assembly favors chromosome segregation defects in human oocytes. Science 348, 1143-1147 (2015).

230. Liu,L. & Keefe,D.L. Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. Hum. Reprod. 17, 2678-2685 (2002).

231. Battaglia,D.E., Goodwin,P., Klein,N.A., & Soules,M.R. Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Hum. Reprod. 11, 2217-2222 (1996).

232. De,S.L. et al. Polar body morphology and spindle imaging as predictors of oocyte quality. Reprod. Biomed. Online. 11, 36-42 (2005).

233. Heindryckx,B., De,G.S., Lierman,S., Gerris,J., & De,S.P. Efficiency of polarized microscopy as a predictive tool for human oocyte quality. Hum. Reprod. 26, 535-544 (2011).

234. Duncan,F.E., Chiang,T., Schultz,R.M., & Lampson,M.A. Evidence that a defective spindle assembly checkpoint is not the primary cause of maternal age-associated aneuploidy in mouse eggs. Biol. Reprod. 81, 768-776 (2009).

235. Tatone,C. et al. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. Biol. Reprod. 74, 395-402 (2006).

236. Cukurcam,S. et al. Influence of follicular fluid meiosis-activating sterol on aneuploidy rate and precocious chromatid segregation in aged mouse oocytes. Hum. Reprod. 22, 815-828 (2007).

237. Wassmann,K., Niault,T., & Maro,B. Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. Curr. Biol. 13, 1596-1608 (2003).

238. Steuerwald,N., Cohen,J., Herrera,R.J., Sandalinas,M., & Brenner,C.A. Association between spindle assembly checkpoint expression and maternal age in human oocytes. Mol. Hum. Reprod. 7, 49-55 (2001).

239. Steuerwald,N.M., Bermudez,M.G., Wells,D., Munne,S., & Cohen,J. Maternal age-related differential global expression profiles observed in human oocytes. Reprod. Biomed. Online. 14, 700-708 (2007).